Studies on a multicatalytic, protease complex from *Trypanosoma brucei brucei*

By

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Preface

The experimental work described in this thesis was carried out in Biochemistry: School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg from June 1995 to June 1999 under the supervision of Dr Theresa H.T. Coetzer and co-supervision of Professor John D. Lonsdale-Eccles. These studies represent original work by the author and have not been submitted in any other form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.

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Abstract

Subcellular fractionation (together with immunocytochemical localisation studies) showed that the parasite *Trypanosoma brucei brucei* possesses a multicatalytic protease complex (MCP-Tb). This complex is predominantly cytosolic but some activity is also present in the nuclear fraction. MCP-Tb was isolated from *T. b. brucei* and compared to the properties of other proteasomes reported in the literature and to the 20S MCP isolated from bovine red blood cells (MCP-rbc). The isolation procedure employed four-steps: anion exchange chromatography on Q-Sepharose, adsorption chromatography on HA-Ultrogel, molecular exclusion chromatography on Sephacryl S-300 and glycerol density gradient sedimentation.

The molecular mass of intact MCP-Tb was shown to be smaller than that of MCP-rbc. Separation of the different proteasome subunits by 2D-PAGE showed that MCP-Tb has 12 different polypeptide components compared to the 28 different polypeptide components of MCP-rbc. The N-terminal sequence of an MCP-Tb subunit showed that this subunit did not have any obvious sequence homology with the subunits of proteasomes from other cells. Furthermore, anti-MCP-Tb antibodies (which exhibited the *in vitro* inhibitory activity of MCP-Tb) did not cross-react with MCP-rbc showing that MCP-Tb and MCP-rbc are antigenically distinct.

The basic enzymatic properties of MCP-Tb were fairly typical of other 20S proteasomes. MCP-Tb had multiple peptidase activities (identified as chymotrypsin-like, trypsin-like and peptidyl glutamylpeptide hydrolase activities) that are characteristic of proteasomes. Furthermore, the characteristics of inhibition by a variety of inhibitors were similar to those of other proteasomes, including MCP-rbc. The activities of 20S proteasomes from most cell types are activated by endogenous high molecular mass complexes such as the bovine 19S complex called PA700. These complexes form end-on associations with the 20S proteasome. However, no endogenous MCP-activator was found in *T. b. brucei*. Nevertheless, MCP-Tb was activated in an ATP-dependent manner by bovine PA700. Inhibition of the intrinsic phosphatase activity of PA700 inhibited the protease enhancing effect of PA700.

Electron microscopic examination of negatively stained MCP-Tb and MCP-rbc showed particles that were morphologically indistinguishable. However, the MCP-Tb also exhibited unique end-on associations between individual units forming long (up to 200 nm) ribbon-like chains. Since access to the active sites of proteasomes occurs through the pores at the end of the complexes, this end-on association, when coupled to our observation of an apparent lack of an endogenous activator, suggests that *T. b. brucei* may have evolved an alternate mechanism for controlling their proteasome activity.

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Last but not least, thanks to the Almighty for the privilege to be alive.

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Abbreviations

A_{280}	absorbance at 280 nm
A_{405}	absorbance at 405 nm
ABTS	2,2'-azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate]
Ac	acetyl
AcP	acid phosphatase
ACTH	adrenocorticotropic hormone
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AFU	arbitrary fluorescence units
AMC	7-amino-4-methylcoumarin
AMT	acetate-Mes-Tris
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AZ	antizyme
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N,N′-methylenebisacrylamide
BrAAP	branched-chain amino acid preferring
BSA	bovine serum albumin
Bz	benzoyl
Cbz (Z)	carbobenzoxy
CDK	cyclin-dependent protein kinases
CY	cytosolic fraction
CysP	cysteine protease
cDNA	complementary deoxyribonucleic acid
CH_2Cl	chloromethylketone
DCI	3,4-dichloroisocoumarine
dd. H_2O	distilled, deionised water
DDT	dichlorodiphenyltrichloroethane
DEAE	diethylaminoethyl
DFMO	α-DL-difluoromethylornithine

DFP	diisopropylfluorophosphate
dist.H_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplamic reticulum
E_{280}	molar extinction coefficient at 280 nm
g	relative centrifugal force
F_{ab}	fragment, antigen-binding
F_c	fragment, crystallisable
Glut	glutaryl
GPDH	glycerol-3-phosphate dehydrogenase
GPI	glycerolphosphatidylinositol
h	hour
Hepes	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HRPO	horse-radish peroxidase
I	ionic strength
IC_{50}	inhibitor concentration inhibiting enzyme activity by 50%
IEF	isoelectric focussing
IgG	immunoglobulin G
IgY	egg-yolk immunoglobulin
ILRAD	now called ILRI (International Livestock Research Institute)
kDa	kilodalton
kDNA	kinetoplast DNA
K_m	apparent Michaelis-Menten constant
l	light-path
LG	large granule

MCP	multicatalytic protease complex
MCP-rbc	multicatalytic protease complex from bovine red blood cells
MCP-Tb	multicatalytic protease complex from *T. brucei*
MDH	malate dehydrogenase
MHC	major histocompatibility complex
MEC	molecular exclusion chromatography
Mes	2-(N-morpholino)ethanesulfonic acid
MI	microsomal fraction
M_r	relative molecular mass
βNA	*beta*-naphthylamide
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	reduced NAD
NBT	nitroblue tetrazolium
N-terminus	amino terminus
NLS	nuclear localisation signal
*p*NPP	*para*-nitrophenyl phosphate
NU	crude nuclear fraction
OP-Tb	oligopeptidase from *Trypanosoma brucei*
ODC	ornithine decarboxylase
PAGE	polyacrylamide gel electrophoresis
PA700	700 kDa protease activator
PA28	28 kDa protease activator
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGPH	peptidylglutamyl peptide hydrolase
pI	isoelectric point
PLT	progressive lowering of temperature
PMSF	phenymethyl sulfonyl fluoride
PSG	phosphate-buffered saline glucose
PSGH	phosphate-buffered saline containing hypoxanthine
PTH	phenylthiohydantoin

PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RT	room temperature
[S]	substrate concentration
SBTI	soya bean trypsin inhibitor
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
SHADE	sucrose-Hepes-ATP-EDTA buffer
SG	small granule fraction
SnAAP	small neutral amino acid preferring
S	Svedberg
TAP1/TAP2	transporter proteins (ER to cytosol)
TBS	Tris-buffered saline
Tb-x	subunit x of MCP-Tb
TCA	trichloroacetic acid
TEMED	N,N,N,'N'-tetramethylethylenediamine
TLCK	tosyl-lysyl-chloromethyl ketone
TPCK	tosyl-phenyl-chloromethyl ketone
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
V_e	elution volume
V_o	void volume
V_{max}	maximum velocity
VAT	variant antigenic type
VSG	variant surface glycoproteins
mfVSG	membrane-form VSG
sVSG	soluble-form of VSG
V_t	total volume
WHO	World Health Organization

Chapter 1

Introduction

1.1 African trypanosomiasis

The African trypanosome is a unicellular protozoan flagellate that spends part of its life cycle as a parasite in the blood and other body fluids of its host. It is the causative agent of a fatal neurological disease, trypanosomiasis, commonly referred to as sleeping sickness in humans and 'nagana' in animals. The debilitating chronic course of the disease is characterised by anaemia and severe endocrinal defects (Lomo *et al.*, 1993; 1995). Trypanosomiasis is endemic in a vast region of Africa defined by the range of the tsetse fly vector. This insect inhabits over a third of the African continent south of the Sahara and it exposes about 50 million people to direct risk of contracting trypanosomiasis. Twenty thousand new cases are reported each year and thousands of other cases go unreported (Kuzoe, 1993). Perhaps of equal importance to the direct threat to human beings is the fact that 30% of the estimated 160 million cattle in the tsetse-affected areas are exposed to the risk of infection (ILRAD report, 1989). The disease makes some four million square miles of Africa uninhabitable for most breeds of dairy and beef cattle. Having little or no access to meat or dairy products, much of the human population is malnourished and susceptible to diseases. Effective control of trypanosomiasis could drastically increase livestock production in Africa, as well as greatly benefit arable farming on the continent, where animals provide most of the traction power in agriculture

Various curative and preventive methods of control, including keeping of trypanotolerant breeds of livestock have been tried, but elimination of the tsetse vector has remained the method of choice in the control of trypanosomiasis. Before the advent of modern insecticides, the clearing of vegetation, on which the flies depend for shelter, and the shooting of wild mammals, on which they primarily depend for food, were widely practised. The introduction of organochlorine insecticides in the late 1940s was followed by large scale spraying campaigns in a number of countries. Many of these operations were effective in reducing the tsetse populations (Jordan, 1995). Nevertheless, there were problems, namely cost, the logistical difficulties of mounting large-scale ground and aerial operations, and the realisation that the operation had to be repeated regularly if disease control was to be maintained. Repeated use of non-biodegradable organochlorine

insecticides resulted in major ecological problems leading to a worldwide ban on the use of dichlorodiphenyltrichloroethane (DDT). Awareness of the need to conserve the environment has rendered the earlier methods of vector control such as bush clearing and killing of wild mammals unacceptable. Furthermore, economies of most countries in the sub Saharan Africa depend on tourism and game viewing forms a major part of tourist attraction to these regions.

Alternative, environment-friendly methods of control of tsetse flies are under investigation. Baited flytraps have been used with considerable success (Putnam, 1993). Another form of control that has been tried with varying degrees of success is the release of sterile insects, particularly sterile male insects into the wild (Williams *et al.*, 1983). A further potential control method is based on the finding that the tsetse fly carries symbiotic bacteria in the cells of its gut and other tissues. Genetic transformation of these bacteria to produce a lectin protein or antibody fragment known to kill trypanosomes before leaving the insect gut was tried with little success (Aldhous, 1993).

Only a very limited number of drugs are available for chemotherapy of African trypanosomiasis. Treatment of this disease in humans is currently dependent upon four drugs: suramin, pentamidine, α-DL-difluoromethylornithine (DFMO) and melarsoprol (Kuzoe, 1993). In cattle, sheep and goats, three compounds (homidium, diminazene and isometamidium) are used to treat nagana (Kinabo, 1993). Regrettably, all these drugs are plagued by various problems ranging from adverse side effects, oral inabsorption, short duration of action, and low efficacies to the emergence of drug resistant strains of trypanosomes (Wang, 1995). In view of the inadequency of the available drugs and considering the dramatic outbreaks of trypanosomiasis in different areas on the African continent, there is a need to identify alternative methods of control. The rational approach is to identify unique biochemical and molecular features of trypanosomes that could be potential chemotherapeutic targets for effective drug design. Thus, it becomes necessary to understand the biological features of the trypanosome.

1.2 Classification of African trypanosomes

The causative agents of African trypanosomiasis are unicellular eukaryotic organisms belonging to the family *Trypanosomatidae* (Fig. 1.1). African trypanosomes share with other *Sarcomastigophora* the characteristic mode of locomotion by flagella. They are also

classified in the superclass *Mastigophora* that contains those protozoans which move by flagella only. Since they do not possess chlorophyll, African trypanosomes are placed in the class *Zoomastigophorea*. They fall into the order *Kinetoplastida* because they possess a kinetoplast, a dense organelle at the base of the flagellum, which contains the mitochondrial DNA. The suborder *Trypanosomatina* is composed of *Kinetoplastida* with only one flagellum and all members appear to be parasitic. African trypanosomes belong to the genus *Trypanosoma* consisting of mammalian-infective species of trypanosomes divided into two groups (stercoraria and salivaria). These two groups differ according to the site where the parasite develops in the vector and the mode of transmission of the infection (Hoare, 1972).

The stercoraria group consists of parasites whose developmental cycle is completed in the "posterior of the vector" and where transmission occurs via the faeces of the vector through skin wounds in the host. *Trypanosoma cruzi*, the South American trypanosome that causes Chaga's disease is the best known species in this section. Reproduction in the animal is discontinuous and usually takes place in the amastigote or epimastigote stage. Stercorarian trypanosomes are not the subject of this thesis and will not be discussed further.

Within the salivarian group of trypanosomes are genera whose developmental cycle in the vector is completed in the anterior (*e.g.* in the salivary gland) of the vector. Transmission from vector to host occurs by the inoculation of the metacyclic stage when the insect bites the new host. The flagella of trypanosomes are attached to the body by means of an undulating membrane but may extend beyond the body of the parasite where the flagella is said to be free. The kinetoplast is relatively small and may be terminal or subterminal. The posterior extremity of the parasite cell body is often blunt. Cell division in the host is continuous in the trypomastigote stage.

In Africa *T. vivax, T. congolense, T. evansi, T. equiperdum* and *T. brucei* are major animal pathogens. *T. vivax, T. congolense* and *T. brucei* are generally transmitted by tsetse flies (*Glossina* species), in which they undergo a strict developmental cycle. However, they can also be directly transmitted by other bloodsucking flies, or by the ingestion of infected carcasses. In contrast, *T. equiperdum* is transmitted venereally between horses.

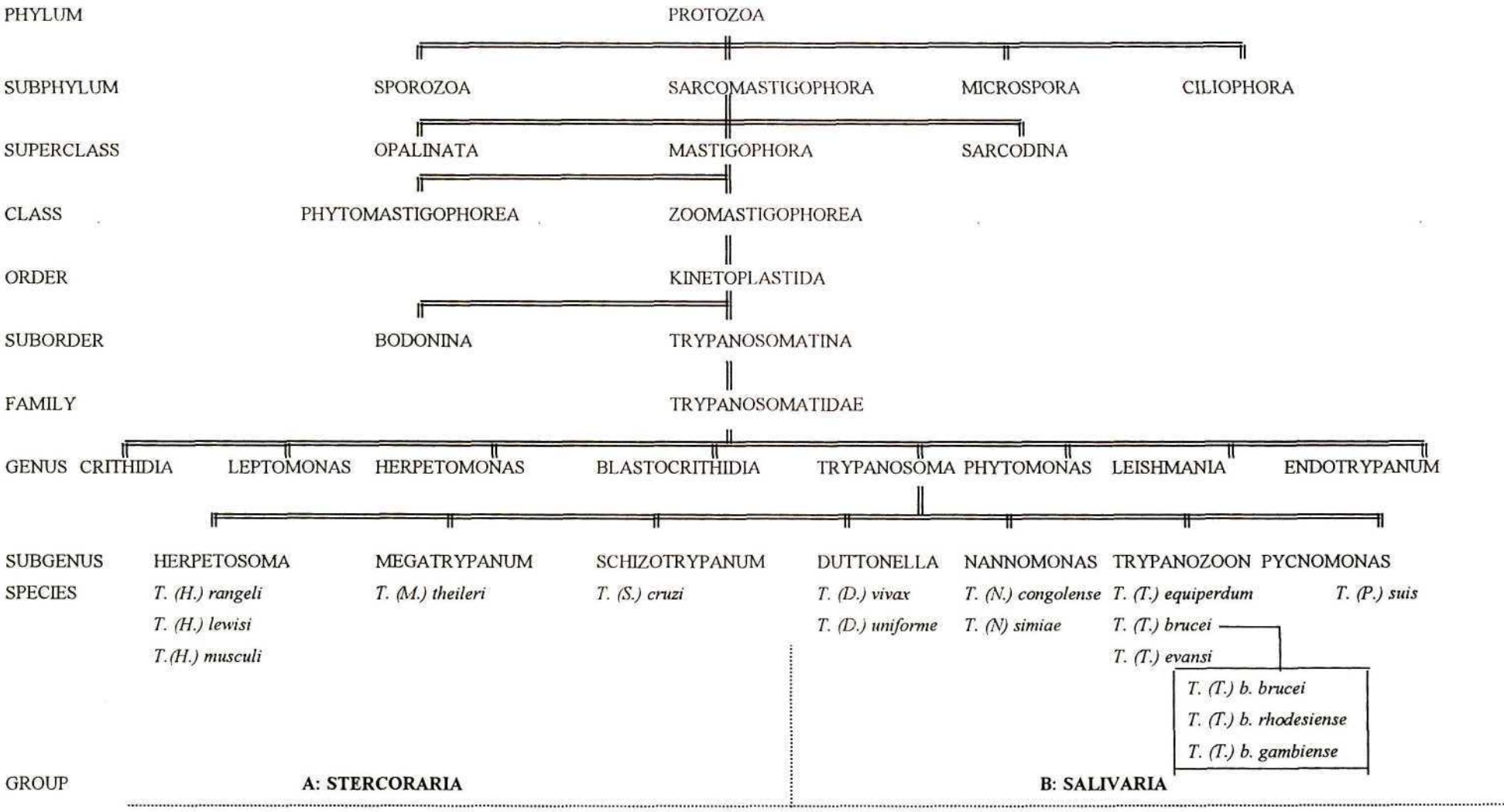

Figure 1.1. Classification of African trypanosomes adapted from Hoare, 1972; Baker *et al.*, 1978

Human sleeping sickness is caused by 'subspecies' of *T. brucei* designated *T .b. rhodesiense* and *T. b. gambiense.* The former is found in Eastern Africa and causes acute illness, whereas *T. gambiense* is found in Western Africa and causes a more chronic disease. *T. b. brucei* is morphologically identical to these two subspecies in all phases of its developmental cycle, but is confined to wild and domestic ungulates and carnivores. It is not generally considered to be infective to man.

1.3 Anatomy of African trypanosomes

The generalised fine structure of *T. brucei* is shown in Fig. 1.2. This parasite is a unicellular organism with a flagellum that stretches along (and may extend beyond) the body of the parasite. The cell body of the trypanosome is bounded by a unit membrane which is coated on its outside by a thick layer of variant surface glycoproteins (VSG). Beneath the limiting membrane run the pellicular microtubules in a longitudinal direction from the anterior to the posterior end of the parasite. The microtubular skeleton maintains the shape and stability of the cell (Hemphill *et al.*, 1991). In addition, *T. brucei* has a precisely ordered microtubule cytoskeleton whose morphogenesis is central to cell cycle events such as organelle positioning, segregation, mitosis and cytokinesis (Robinson *et al.*, 1995).

The flagellar pocket of an African trypanosome is a flask-like invagination at the posterior end of the cell body where the flagellum leaves the surface of the parasite and enters into the main body of the cell. The flagellar pocket membrane is devoid of subpellicular microtubules. The flagellar pocket constitutes 0.2-1% of the total cell volume (Webster and Fish, 1989), has an inner surface of about 1μm^2 (Coppens *et al.*, 1988) and is considered to be an important site of endocytosis (Langreth and Balber, 1975; Webster and Russel, 1993). There is also evidence, from freeze-fracture analysis of the trypanosomatidae *Leptomonas collosoma* (Fig. 1.1), for a role of the flagellar pocket in exocytosis (Linder and Staehelin, 1979). The plasmalemma of the cell body is continuous with that of the flagellum through the flagellar pocket.

The trypanosome flagellum begins from a structure called the basal body or kinetosome found below the floor of the flagellar pocket. The flagellum contains two major structural components: the axoneme and the paraflagellar rod (Schlaeppi *et al.*, 1989). The axoneme is formed by the characteristic 9+2 associated microtubular structures. It consists of nine

doublet microtubules held in a ring by interdublet linkages termed nexin links. This ring surrounds a central core of two singlet microtubules. The flagellum forms an undulating membrane that runs along the pellicle and is attached to the trypanosome along the whole length of the body.

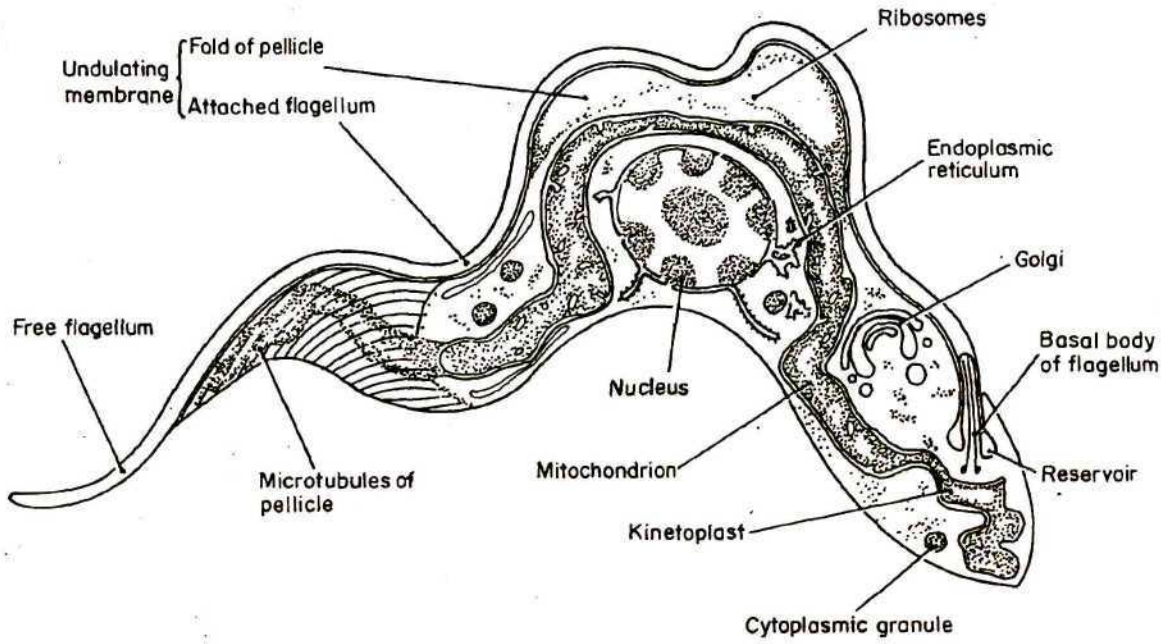

Figure 1.2. Schematic diagram of bloodstream form of *Trypanosoma brucei* illustrating the major organelles (adapted from ILRAD 1989 Annual Report)

At the opening of the pocket where the flagellum emerges from the cell body, there is a thickening of the flagellum and the appearance of 'hemidesmosomal' junctions within the flagellum and the opposing region of the cell body. These regions are thought to limit the access of materials to the lumen of the pocket area or act as anchor points for the flagellum to the cell body (Webster and Russel, 1993). The flagellum may terminate at the anterior end of the cell body (*T. congolense*) or it may extend beyond the cell body when it is said to become "free" (*T. brucei*). In bloodstream forms, in the vertebrate host, the flagellum is responsible for movement while in the invertebrate it plays a dual role; locomotion and attachment.

The main body of the parasite contains various intracellular organelles analogous to those in other cells. The mitochondrion of *T. brucei* is unusual in that it is a single elongated tubular structure that extends along the length of the cell (Hoare, 1972). The

mitochondrion is more developed in the tsetse fly stages and less developed in the bloodstream form stages, where energy is readily available in the form of glucose in the blood of the host (Vickerman *et al.*, 1988). The mitochondrial genome is not distributed throughout this single mitochondrion, but instead exists within the mitochondrion as a discrete physical structure called the 'kinetoplast'. The kinetoplast DNA (kDNA) consists of maxicircle and minicircle components. The maxicircle components play a role in the cycle of mitochondrial activation and repression and are considered the true mitochondrial DNA encoding several respiratory enzymes. The more numerous minicircles, which appear to hold the maxicircles together in a network, are thought to serve as guides for RNA editing or the transcripts of the mitochondrial genome (Stuart, 1991).

The nucleus of trypanosomes may be spherical or slightly ovoid and it may be situated in one of the several different parts of the parasite body. In bloodstream form parasites, it is normally situated in the centre of the anterior half of the cell (Hoare *et al.*, 1972). As in all other eukaryotes the nucleus is bound by a double membrane envelope with pores. The outer nuclear membrane is continuous with the rough endoplasmic reticulum which is associated with the Golgi apparatus, subpellicullar microtubules and mitochondrial network (Hemphill *et al.*, 1991). The nucleolus lies in the center of the nucleus and the condensed chromatin is found in the nucleoplasm especially immediately beneath the nuclear envelope.

Trypanosomes possess unusual organelles called glycosomes. These contain glycolytic enzymes normally located in the cytosol in other eukaryotic cells (Opperdoes *et al.*, 1987). Glycolysis is essential for African trypanosomes living in the bloodstream of the mammalian host because it is their sole source of energy. They lack a functional Kreb's cycle and mitochondrial ATP-synthesising respiratory chain and therefore entirely depend on the aerobic conversion of glucose to pyruvate (Bowman and Flynn, 1976). Pyruvate is excreted as a metabolic end product into the bloodstream.

The endosomes and lysosomes of trypanosomes are specialised structures, similar to mammalian endosomes and lysosomes that contain hydrolytic enzymes (including proteases and phosphatases) that break down endocytosed molecules (Lonsdale-Eccles and Grab, 1987). Newly formed intracellular vesicles that have detached from the flagellar pocket membrane travel into the cell where they fuse with endosomes and deposit their

ingested material. This ingested material passes through the endosome [where protein sorting is also thought to occur (Webster and Fish, 1989)], into lysosomes where the intracellular digestion is completed. Proteases such as trypanopain-Tc from *T. congolense*, which have been localised in lysosome-like organelles, are thought to digest endocytosed materials (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, 1992). The breakdown products such as amino acids then pass through the lysosomal membranes into the cell's cytoplasm, where they can be used to synthesise new proteins (Ferguson and Williams, 1988).

Other cytoplasmic membrane systems include the Golgi apparatus, which is found between the nucleus and the flagellar pocket, where glycosylation and packaging of the newly synthesised proteins (including hydrolytic enzymes) occur (Bangs *et al.*, 1986; Ferguson *et al.*, 1986). The endoplasmic reticulum may be rough containing attached ribosomes or smooth without the associated ribosomes. These play a similar role as in other eukaryotes, transporting proteins to the Golgi apparatus (Webster, 1989; Clayton *et al.*, 1995).

1.4 Life cycle of African trypanosomes

Development in the insect vector

Trypanosomes, like many other parasites, assume different forms at different stages of their complex developmental cycle (Fig. 1.3) (Vickerman, 1985; Vickerman *et al.*, 1988). The parasite shape, the position of the kinetoplast in relation to the nucleus, and the extent of the flagellar apparatus identify the distinct morphological stages. For example, the trypomastigote (trypanosome) stage has a post-nuclear kinetoplast and body-attached flagellum, while the epimastigote (crithidial) stage has a prenuclear kinetoplast and similar flagellum, and the rounded immobile amastigote (leishmanial) stage lacks an emergent flagellum. In *T. brucei*, two distinct morphological forms of trypanosomes are seen in the bloodstream. Slender (dividing) forms predominate early in the infection while stumpy (non dividing) forms are seen at declining parasitaemia (Vickerman, 1965; Hecker *et al.*, 1973)

The life cycle of *T. brucei* may be arbitrarily considered to begin when trypanosomes are ingested by a feeding tsetse fly. The ingested parasites lodge in the fly's midgut, where the stumpy trypanosomes (which are insect infective) transform into the so-called procyclic stage. The slender forms die or change into stumpy forms in the anterior midgut. Transformation to procyclic forms takes place in the posterior part of the midgut in the

endoperitrophic space. Morphological transformation is accompanied by an increase in body length, elongation of the post-kinetoplastic portion of the body and repositioning of the mitochondrial genome (Mathews *et al.*, 1995). Concomitantly, glycosomes change from spherical to bacilliform structures. The coat of variable antigen is progressively lost and endocytosis diminishes (Steiger, 1973).

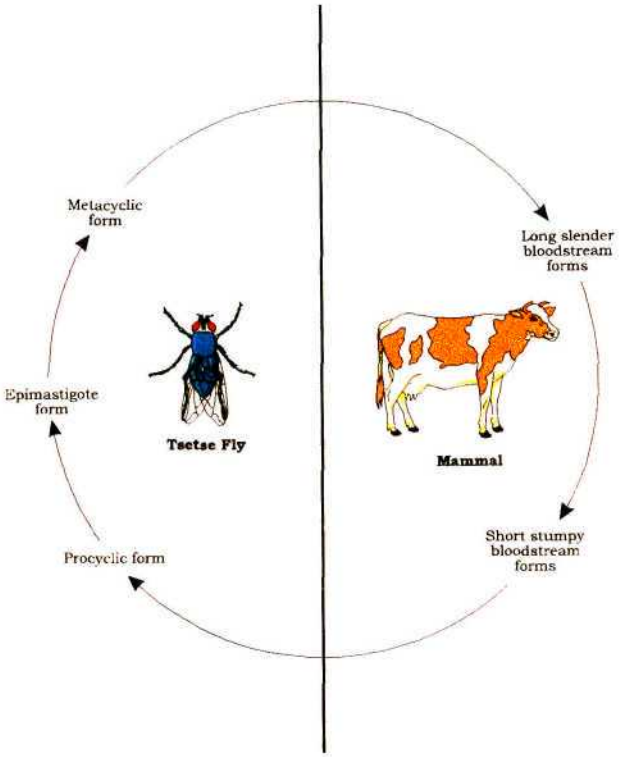

Figure 1.3. Schematic diagram showing the life cycle of *T. brucei* (adapted from Vickerman, 1985)

Procyclic development is associated with a switch from the use of glucose to that of proline as the principal energy source. Interestingly, the tsetse itself uses proline as an energy source for flight (Bowman and Flynn, 1976). The procyclic trypomastigote forms migrate to the salivary glands where they become anchored by their flagella to the tsetse fly epithelial cells. Here they multiply as a distinct morphological stage called the epimastigote. These epimastigote forms have no obvious surface coat, and can divide. After cessation of division, the epimastigote starts to express the variant surface glycoprotein (VSG) coat once more (Tetley *et al.*, 1987). This expression leads to detachment and maturation as nondividing, infective, metacyclic trypomastigotes. The VSG coat is a unique feature that facilitates the survival of the trypanosomes in their mammalian host and will thus be discussed further in the subsequent section.

Development in the mammalian host

When an infected tsetse fly feeds, it injects metacyclic trypomastigotes into the dermal tissues of the host. Here a local inflammatory reaction, the 'chancre', develops (Roberts *et al.*, 1969). From the chancre the parasites migrate to the lymphatic system and the bloodstream. They may traverse into connective tissues through the walls of blood and lymph capillaries. Eventually, they cross the choroid plexus into the brain and cerebrospinal fluid. In all these sites they multiply by binary fission with a doubling time of about 6 hours in the case of *T. b. gambiense* (Seed, 1978). Because of the growth characteristics of the parasites, and because of their interplay with the host's immune system, parasitaemia fluctuates in a cyclical manner that corresponds to the growth of different antigenic variants of the parasites. During ascending parasitaemia, parasites with a particular antigenic type (called the 'homotype') form the major part of the population (Van Meirvenne *et al.*, 1975). When the host mounts an IgM response to the homotype, parasitaemia goes into remission as trypanosomes of that variable antigenic type (VAT) are killed (Seed, 1977)

In the case of *T. brucei*, the rapidly dividing bloodstream forms of the parasites are long and slender. During the latter stages of a parasite growth-curve, the parasites stop dividing and become short and stumpy. At this point parasitaemia goes into decline. It is believed that short stumpy trypomastigotes can only continue the life cycle in the vector.

In addition to the homotype VAT, trypanosome populations contain minor VATs or 'heterotypes' that continue to multiply during remission (Van Meirvenne *et al.*, 1975). One of these VATs overgrows the others to give rise to a recrudescence of parasitaemia in which it becomes the homotype. Again, parasitaemia peaks and goes into remission, and so the process continues, to give the chronic, cyclic infection characteristics of African trypanosomes.

1.5 Antigenic variation

The key to the trypanosome's success is its ability to circumvent the mammalian immune system. A mammal ordinarily defends itself against viruses, bacteria or protozoa such as trypanosomes by producing specific antibodies directed against antigens it recognises on the surface of the foreign organism. The antibodies bind to the antigens and either neutralise the effects of the invading organisms or help to destroy them. Some antibody-

producing cells persist in the bloodstream and provide lasting immunity. A vaccine that mimics a natural infection can also elicit such immunity. A humoral response is thought to be more effective against organisms that invade the bloodstream as in malaria or African trypanosomiasis, whereas cell-mediated immunity is the effective immune response against parasites that grow within tissues (e.g. cutaneous Leishmaniasis or *T. cruzi* infection) (Taylor, 1998).

Trypanosomes evade the host's specific immune response by changing their surface glycoprotein. These variant surface glycoproteins or VSGs, form a 15 nm coat which overlays the plasma membrane of the cell body and the flagellum of the bloodstream (and metacyclic) forms of the parasites (Vickerman, 1969). The change in VSG coat, termed antigenic variation, is a process that involves sequential synthesis and surface expression of different VSGs during the course of infection (Cross, 1990; Vickerman, 1978). By the time the immune system has made new antibodies to bind to the new antigens, some of the trypanosomes have shed their coat and replaced it with yet another that is antigenically distinct. Eventually, the host's overworked immune system becomes unable to cope with this or other infections and the host succumbs.

Each VSG is encoded by a separate gene (Steinert and Pays, 1985). Changes of the VSG coat are initiated by transcriptional activation of a new VSG gene (Pays *et al.*, 1994). The VSGs are synthesised in the rough endoplasmic reticulum, a process that takes 6 to 8 min (Grab *et al.*, 1984). The VSGs are then glycosylated and a glycosylphosphatidyl-inositol (GPI) anchor added (Cross, 1990; Furguson and Williams, 1988). The VSGs are transported to the Golgi apparatus and finally to the cell surface. The whole process takes about 45 min (Bangs *et al.*, 1986; Ferguson *et al.*, 1986).

The expression of VSG is not only regulated throughout the life cycle of the parasite, but it is also modulated during trypanosomal development in the blood. For example, non-dividing trypanosomes (e.g. stumpy forms) do not undergo antigenic variation (Vickerman, 1985). Trypanosomes expressing different VSGs appear successively during chronic infection. Although the repertoire of the VSGs appears unlimited, the timing of its expression is not entirely random. Some VSGs are usually observed early in the infection, while others emerge only later.

Two forms of VSG have been isolated; a membrane-form (mfVSG) (Cardoso de Almeida and Turner, 1983) and a hydrophilic, water-soluble form (sVSG) (Cross, 1975). The mfVSG is readily converted to sVSG, during extraction procedures, by a hydrolytic process catalysed by mfVSG hydrolase, an endogenous phospholipase C-like enzyme (Bulow and Overath, 1985). The conversion of mfVSG to sVSG is stimulated upon parasite lysis, by Ca^{2+} in the presence of ionophores or benzyl alcohol (Jackson *et al.*, 1985). However, mfVSG is not hydrolysed to sVSG so long as membrane integrity is maintained. Interestingly, a localisation study indicated that the GPI-specific phospholipase C in trypanosomes is found on the cytosolic face of vesicles and not at the cell surface, and as such may not be directly responsible for VSG release *in vivo* (Bulow *et al.*, 1989).

Curiously, in order to purify mfVSG, protease inhibitors e.g. 1 mM tosyl-lysyl-chloromethyl ketone (TLCK) and 0.2 mM phenylmethyl sulfonyl fluoride (PMSF) are required to stop conversion of mfVSG to sVSG (Cardoso de Almeida *et al.*, 1984). Since these reagents are potent inhibitors of trypsin-like and chymotrypsin-like activities, it is likely that proteases play a role in this conversion. Indeed, studies on the release of VSG indicates that proteolysis is involved (Ziegelbauer *et al.*, 1993).

1.6 Pathophysiological role of proteases

Proteases are important constituents of all eukaryotic organisms. In the case of protozoan parasites, some of these enzymes are thought to assist the parasites by helping them to evade the host immune system; for example, by degrading the antibodies directed against the parasite (Russo *et al.*, 1993, 1994; Donelson *et al.*, 1998). They may also help the parasites to invade host tissues or cells e.g. *T.cruzi* invasion of fibroblasts (Burleigh and Andrews, 1995).

Since African trypanosomes are generally considered to be extracellular parasites, their proteases are unlikely to be involved in cell invasion processes, although they may well play roles in the penetration of different tissues. Indeed, there is evidence that African trypanosomes release proteases into the host's bloodstream. Here the proteases may adventitiously induce pathology by degrading host proteins and peptidyl hormones (Nwagwu *et al.*, 1988; Boutignon *et al.*, 1990; Troeberg *et al.*, 1996). In some cases, these proteases are rapidly inhibited by endogenous protease inhibitors in the blood (e.g. the

trypanosome-released lysosomal cysteine proteases by cystatins) but in other cases they are not (e.g. the trypanosome-released cytosolic serine endopeptidase called OP-Tb by serpins) (Troeberg *et al.*, 1996). Thus, unihibited, released proteases such as OP-Tb may play important roles in disease pathology. However, such actions are probably secondary to their nutritional and intracellular maintenance functions within the parasites themselves.

One proteolytic system that has been shown to be essential to the proliferation and cell cycle transformation of trypanosomes, is the proteasome. Proteasome activity is required for the transformation of *T. cruzi* trypomastigotes (non-replicative bloodstream forms) into amastigotes (obligate intracellular replicative forms) since lactacystin or peptide aldehyde MG132, both of which are inhibitors of the proteasome activity, prevents the intracellular stage-specific transformation of these parasites (Gonzalez *et al.*, 1996). Similarly, inhibition of proteasome activity also blocks cell cycle progression in bloodstream and procylic forms of *T. brucei* (Mutomba *et al.*, 1997). Thus, it appears that proteasome-mediated proteolysis is essential for the transformation and proliferation of trypanosomes. Indeed, the two events may be tightly linked by proteasome activity. One possible explanation of these observations is that the proteasome regulates cell division through degradation of cyclins that act in different phases of the cell cycle (Glotzer *et al.*, 1991; Affranchino *et al.*, 1993).

1.7 Characterisation and classification of proteases

Proteases are ubiquitous enzymes that catalyse the degradation of peptide bonds in peptides or proteins. Their active sites consist of a catalytic site and substrate-binding subsites. The latter are located on either sides of the point of cleavage of the substrate in the active site cleft (Schechter and Berger, 1967). By convention, these specificity subsites are numbered S_1, S_2, S_3....S_n, away from the catalytic site towards the N-terminus of the substrate, and S_1', S_2', S_3'...S_n' towards the C-terminus (Fig. 1.4). The corresponding amino acid residues of the substrate fitting into these pockets are designated as P_1, P_2, P_3 ....P_n on the N-terminal and P_1', P_2', P_3'....P_n' on the C-terminal of the peptide bond being cleaved, respectively.

There are two major types of proteases (peptidases): endopeptidases and exopeptidases (Barrett and Rawlings, 1991). Endopeptidases are also known as proteinases and catalyse the cleavage of an internal peptide bond within a protein. Exopeptidases catalyse the

cleavage of one or two amino acids from the extreme amino terminus (aminopeptidases) or carboxyl terminus (carboxypeptidases) of a protein or peptide.

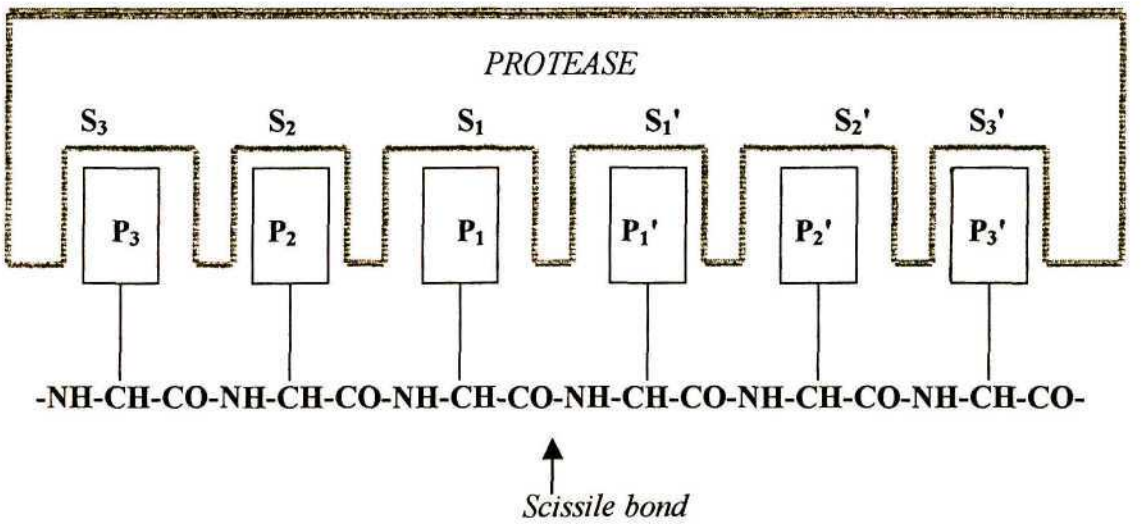

Figure 1.4. Schematic diagram of the subsite specificity of a protease.

P_1.....P_1' are the side chains of six substrate amino acids, and S_1...S_2' are the corresponding subsites on the enzyme (Schechter and Berger, 1967)

Typically proteases are classified into five distinct groups on the basis of the chemical groups that are responsible for their catalytic activity (Fig. 1.5). They are the serine, cysteine, metallo-, aspartic, threonine and serine proteases (Barrett and Rawling, 1991; Seemuller *et al.*, 1995). These groups of proteases are most easily distinguished by appropriate type-specific inhibitors. The endopeptidases in each of these five groups are further categorised into families on the basis of 'evolutionary line'.

The term 'family' refers to a group of peptidases in which the amino acid sequence of the part of the protein responsible for catalytic activity shows a statistically significant relationship to that of at least one other member (Barrett and Rawling, 1995). Members of the same family are homologous and are believed to have a single evolutionary ancestor. Such indications come primarily from the linear order of catalytic site residues in the polypeptide chain and the tertiary structure of the protein.

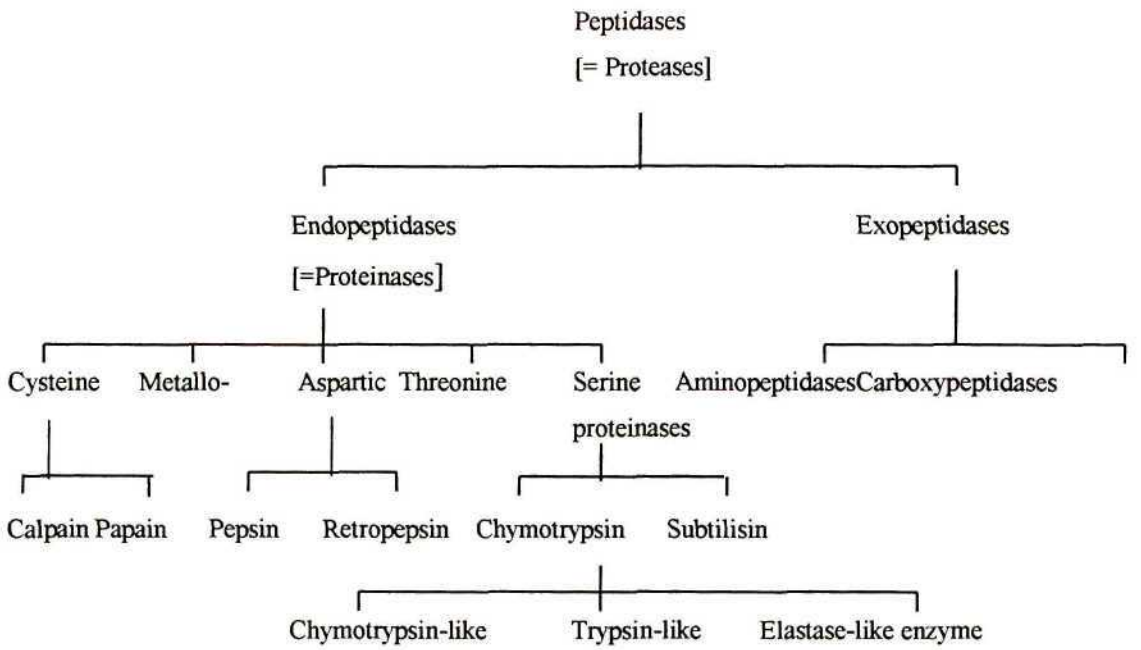

Figure 1.5. Scheme of nomenclature of proteases adapted from Barrett and Rawling, 1991 and Seemuller *et al.*, 1995

1.7.1 Metalloproteinases

The metalloproteinases are widespread in bacteria, fungi, and in higher organisms. Their active site clefts contain a metal ion that plays a key role in substrate binding and catalysis. For example, in thermolysin (from *Bacillus thermoproteolyticus*) a zinc atom held in position by two closely spaced histidine side-chains, and a more remote glutamate residue (Bode *et al.*, 1992) are crucial for catalytic activity. Thermolysin also has four binding sites for Ca^{2+}, but this ion is important for enzyme stabilisation rather than catalytic activity.

The catalytic activity of metalloproteinases is usually inhibited by metal ion chelators such as EDTA. These enzymes typically show maximal activity at about pH 7.0. They seldom cleave bonds other than true peptide bonds between amino acid residues. These residues typically have non-polar side chains, and often a bulky hydrophobic residue such as leucine or phenylalanine is favoured in P_1. Many of the mammalian metalloproteinases are secreted from cells as zymogens that can be activated by limited proteolysis. Most families of metalloproteinases have a conserved –His-Glu-Xaa-Xaa-His (or 'HEXXH)

sequence that is thought to be involved in binding zinc at the active site cleft (Becker and Roth, 1992). Included in this group are carboxypeptidase A and B from mammalian pancreas, astacin from the crayfish digestive gland, membrane-bound metalloproteinases of *Leishmania* (Etges and Bouvier, 1993) and African trypanosomes (El-Sayed and Donelson, 1997). The membrane-bound metalloproteinases may be involved in extracellular digestion of host proteins for parasite nutrition or to assist in the penetration of host cells and tissues e.g. penetration of the walls of blood and lymph capillaries by the trypanosomes.

1.7.2 Aspartic proteinases

Aspartic proteinases have been found in all studied organisms. However, they are scarce in bacteria. Most of the fungal and mammalian aspartic proteinases have pH optima between 2.5-4.5. The important catalytic site residues of these enzymes are two carboxyl groups contributed by a pair of aspartic acid residues. The aspartic proteinases act best on peptide bonds between bulky hydrophobic amino acid residues such as Leu-Tyr, Tyr-Leu, Phe-Phe and Phe-Tyr bonds. The fungal aspartic proteinases have been reported to have relative molecular masses in the range of 30 000-45 000 and isoelectric points below pH 5.1 (North, 1982). There are two major groups of aspartic proteinases: the pepsin and retropepsin families.

1.7.2.1 Pepsin family

The pepsin family includes pepsin and chymosin found in the mammalian stomach, plasma renin whose protein substrate is angiotensinogen, and the lysosomal cathepsins D and E. The gastric proteinases of higher animals are secreted as zymogens that are converted to active enzymes by proteolytic cleavage of an N-terminal extension of the propolypeptide chain. The limited proteolysis is initiated at acid pH by the zymogens themselves. Pepsin A is irreversibly inactivated at and above neutral pH (i.e. when it leaves the stomach and enters the small intestine). Enzymes of the pepsin family are bilobed molecules with the active site cleft located between the lobes and each lobe contributing one of the pair of aspartic (Asp^{32} and Asp^{215}) residues that is responsible for catalytic activity (Rawlings and Barrett, 1993). Most aspartic proteinases are inhibited by their peptide substrate analogue pepstatin.

1.7.2.2 Retropepsin family

In contrast to pepsin, retropepsin consists of a single lobe and carries only one catalytic aspartic acid residue, so that formation of the active enzyme requires the formation of a non-covalent homodimer. The retropepsin family includes the retroviral endopeptidases such as those of the human immunodeficiency virus (HIV). These enzymes play an essential part in the viral life cycle. For example, an aspartyl proteinase, HIV-1 PR is required for proper protein processing and production of infectious virions (Seelmeier *et al.*, 1988). Recently, protease inhibitors have been introduced as a part of the regimen for the treatment of HIV-infected patients. Among the parasitic protozoa, aspartic proteases has been reported in *Plasmodium falciparum* (Vander jagt *et al.*, 1989). The acid pH optimum implies a lysosomal localisation of aspartic proteases in parasitic protozoa. Thus aspartic proteases may be involved in the lysosomal intracellular degradation of proteins in the parasite but this has not been demonstrated.

1.7.3 Cysteine proteinases

Cysteine proteinases are found in bacteria, eukaryotic microorganisms, higher plants and animals. Many occur within the intracellular vacuoles of plant cells and lysosomes of animal cells. Cysteine proteinases are recognized by their sensitivity to inhibition by thiol blocking agents. Although trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (Sreedharan *et al.*, 1996) is generally regarded as the definitive cysteine proteinase inhibitor, many inhibitors of peptidases of other catalytic types can also inhibit cysteine proteinases. These inhibitors include organomercurial compounds, leupeptin, chymostatin and antipain (Bontempi *et al.*, 1984). There are two superfamilies of cysteine proteinases: papain-like and calpains.

1.7.3.1 Papain family

Papain is the standard against which most cysteine proteases are compared. It is a cysteine proteinase from the latex of the plant *Carica papaya.* There are two branches of the papain superfamily; one branch includes mammalian cathepsins L and H, and the plant proteinases; the other branch contains mammalian cathepsin B and cysteine proteinases thus far found in helminths (Rawlings and Barrett, 1993). The enzymes range in size from 212 amino acid residues (papain, 23.4 kDa) to 252 amino acid residues (cathepsin B, 29.0 kDa). Cysteine proteinases contain a thiol-imidazolyl system within the active site. The active site residues involved in catalysis are Asn^{157}, Cys^{25} and His^{159} (papain numbering).

Catalysis proceeds via a thiol ester formed transiently between the substrate and Cys^{25}. Side chains of adjacent residues, including His^{159} facilitate ester bond formation. Cysteine proteinases tend to have a broad specificity with respect to protein and peptide sequences, but may show a preference for arginine at the P_1 position and an arginine or a hydrophobic residue at the P_2 position.

Cysteine proteinases with cathepsin L-like activity have been studied in a number of parasitic protozoa including *Entamoeba histolytica* (Reed *et al.*, 1989), *Leishmania mexicana* (Coombs *et al.*, 1991), *Plasmodium* spp. (Rosenthal *et al.*, 1991) and epimastigotes of *T. rangeli* (Labriola and Cazzulo, 1995). In bloodstream trypomastigotes of *T. brucei* (Lonsdale-Eccles, 1991; North, 1991), a cathepsin L-like activity has been reported to play a role in degradation of internalised anti-VSG antibodies (Mbawa *et al.*, 1991a; Russo *et al.*, 1993). Like most cysteine proteinases, trypanopain-Tc from *T. congolense* is most active at slightly acid or neutral pH. For maximum activity *in vivo* it requires the presence of reducing agents such as dithiothreitol (Mbawa *et al*, 1991a, 1992).

Micromolar concentrations of peptidyl cysteine proteinase inhibitors arrest parasite replication and trypomastigote-amastigote differentiation (Ashall, 1990; Mereiles *et al.*1992; Harth *et al.*, 1993; Scory *et al.*, 1999; Troeberg *et al.*, 1999). In addition, cysteine proteinase inhibitors prevent the normal autocatalytic processing and trafficking of a cysteine proteinase within the Golgi apparatus in *T. cruzi* (Engel *et al.*, 1998). Thus, cysteine proteinases of trypanosomes are potential targets for chemotherapy in trypanosomiasis.

1.7.3.2 Calpain family

The calpain family of cysteine proteinases is comprised of calcium-dependent cysteine proteinases that show maximal activity at about pH 7.8. They are widely distributed in invertebrate and vertebrate animals where they occur mainly in the cytosolic fraction of cells. The calpains exist as heterodimers comprising two subunits of 80 and 30 kDa. The large subunit is homologous to papain and contains a segment (towards the N-terminus) responsible for the proteolytic activity of the molecule. The 30 kDa subunit plays a regulatory function. The C-terminal segments of both subunits show homology with the calmodulin family of calcium-binding proteins, containing calcium-binding sites similar to those of the calmodulins. The Ca^{2+} in the calpains is thought to

function by mediating a conformational change in the active site as opposed to the Zn^{2+} atom in metalloproteinases that function directly in the catalytic mechanism. Calpains are specifically inhibited by calpastatin. Calpastatin is thought to inhibit the activity of calpain by chelating the Ca^{2+} ion. Although a protease of the calpain family has not been isolated from trypanosomes, a conserved sequence has been reported suggesting the presence of a calpain-like molecule in *T. b. rhodesiense* (El-Sayed, *et al.*, 1995).

1.7.4 Serine proteases

Serine proteinases are the most numerous, diverse and widely studied endopeptidases. They are found in prokaryotic and eukaryotic microorganisms, and in vertebrate and invertebrate animals. They are so named for the amino acid serine, whose hydroxyl group is involved in the binding and catalysis of the substrates. The active site of serine proteinases consists of a catalytic triad comprising an aspartate, a histidine and a serine residue. There are two major families of serine endopeptidases, the chymotrypsin and subtilisin families (Barrett and Rawlings, 1995). Serine proteases are readily inhibited by both natural and synthetic inhibitors such as α_2- macroglobulin, α_1-antichymotrypsin, α_1-antitrypsin, aprotinin, chymostatin, leupeptin, antipain and chloromethylketones. However, a diagostic test for serine proteinases is the inhibition by DFP that inhibits the enzyme by affinity labelling the serine residue at the active site.

1.7.4.1 Chymotrypsin family

The catalytic triad in chymotrypsin-related proteinases is arranged in the order His/Asp/Ser and they are inhibited by diisopropylfluorophosphate (DFP). The chymotrypsin-related enzymes can be grouped into three categories on the basis of their primary subsite specificity: chymotrypsin-like, trypsin-like, and elastase-like enzymes (Rawlings and Barrett, 1993; Barrett and Rawlingss, 1995). Chymotrypsin-like enzymes primarily hydrolyse peptide bonds at the carboxyl group of hydrophobic amino acid residue (e.g. tryptophan or phenylalanine). Trypsin-like enzymes have specificity for basic side chains (e.g. arginine and lysine) in the P_1 position and elastase-like enzymes exhibit a preference for small aliphatic residues (e.g. alanine) in the P_1 position. An example in this group is trypsin-like oligopeptidase (OP-Tb) that has recently been isolated from *T. brucei* (Kornblatt *et al.*, 1992; Morty *et al.*, 1998). It has been demonstrated that *T. brucei* releases OP-Tb, and it has been postulated that this enzyme may induce pathology during

infection by degrading host peptidyl hormones (Troeberg *et al.*, 1996) because the protease inhibitors normally present in blood (serpins) fail to inhibit the activity of OP-Tb.

1.6.4.2 Subtilisin family

Members of the subtilisin family are widely distributed in microorganisms, plants and higher animals. The subtilisins have the same triad of catalytic residues as the chymotrypsin family but in the order Asp/His/Ser in the polypeptide chain (Barrett and Rawlings, 1991, 1995). However, the tertiary structures of chymotrypsin and subtilisin families have quite different folding patterns. Typical bacterial subtilisin contains no cysteine, and thus no disulfide bonds. Thermitase from *Thermoactinomyces vulgaris* contains a single cysteine residue close to the catalytic histidine. This is retained in many eukaryotic homologues such as proteinase K and yeast proteinase B (Moehle *et al,* 1987). This thiol group can react with some thiol-reactive reagents, so that the enzymes are inhibited, much as if they were cysteine endopeptidases. Most subtilisins require Ca^{2+} ions for activation and stabilisation. Subtilisin homologues from yeast to man function in the processing of precursor proteins. For example, the cleavage at paired basic residues that occurs in the maturation of numerous mammalian proteins including insulin and the adrenocorticotropic hormone (ACTH)-related hormones. There are no reports available on proteases of the subtilisin family among the parasitic protozoa.

1.6.5 Threonine proteinases

The catalytic mechanism of the proteasome (Löwe *et al.*, 1995; Seemuller *et al.*, 1995) is distinct and different from the typical 'serine', 'cysteine', 'aspartic' and 'metallo'-proteinases (Barrett, 1980). Active site-directed mutagenesis experiments with a recombinant proteasome from *Thermoplasma* have shown that the proteasome represents a new class of proteases referred to as 'threonine' proteases (Seemuller *et al.*, 1995). The protease is a high molecular mass multicatalytic protease complex (MCP) that forms an essential component of the intracellular, ATP-dependent proteolytic pathway. The term 'proteasome' was proposed by Arrigo *et al.* (1988) to indicate the proteolytic and particulate nature of the protein. The proteasome is responsible for the degradation of most cellular proteins (Rock *et al.*, 1994) and is necessary for cell viability (Tanaka, 1995). This particle is the major neutral proteolytic activity in mammalian cells and constitutes up to 1% of the cell protein (Tanaka *et al.*, 1986). However, its concentration varies

considerably among cell types and is greater in organs (e.g. liver) in which average rates of protein breakdown are higher than in other tissues such as muscle (Tanaka *et al.*, 1986).

Proteasomes are present in the nuclei, microsomes and cytosol of all eukaryotic cells examined (Rivett *et al.*, 1992; Peters *et al.*, 1994). Specific examples are *Saccharomyces cerevisiae* (Groll *et al.*, 1997), *Entamoeba histolytica* (Scholze *et al.*, 1996) *T. cruzi* (Gonzalez *et al.*, 1996) and in *T. brucei* (Hua *et al.*, 1996; To and Wang, 1997; Lomo *et al.*, 1997). Some particles are also found associated with the endoplasmic reticulum (Palmer *et al.*, 1996). The proteasome has been reported in prokaryotic cells including *Rhodococcus erythropolis* (Tamura *et al.*, 1995) and *Thermoplasma acidophilum* (Seemuller *et al.*, 1995).

There are two distinct forms of the particle, the 20S (700 kDa) and the 26S (2000 kDa) proteasome complexes. These forms of the proteasome may be distinguished by the ability of 20S proteasome to degrade certain denatured or oxidized proteins in the absence of ATP *in vitro,* whereas the 26S complex requires ATP for activity (Tanaka, 1998). Structurally, the larger 26S complex consists of the 20S proteolytic core and approximately 20 additional polypeptides found in a distinct complex called the 'PA700 proteasome activator', or the '19S complex' (Chu-Ping *et al.*, 1994). These individual complexes will now be considered in greater detail in the subsequent sections.

1.7.5.1 20S Proteasome

The 20S proteasome is a cylinder-shaped particle composed of four stacked rings. It has a diameter of approximately 11 nm, a length of about 15 nm and an internal cavity (Lowe *et al.*, 1995; Groll *et al.*, 1997). This particle contains multiple subunits ranging in size between 20-35 kDa and has a molecular mass of 700 kDa. The 20S proteasome of archaebacterium, *Thermoplasma* strongly resembles the eukaryotic 20S proteasome in its quarternary structure. However, the proteasome from *Thermoplasma* contains only two distinct types of subunits, α and β (Koster *et al.*, 1995) (Figure 1.6). Electron microscopic and X-ray crystallographic analyses have shown that its two outer rings contain only α subunits, and its two inner rings only β subunits (Lowe *et al.*, 1995). The particle has 7 subunits per ring arranged in the order $\alpha_7\beta_7\beta_7\alpha_7$ (Coux *et al.*, 1996). A recent report (Groll *et al.*, 1997) shows that the yeast (*S. cerevisiae*) 20S proteasome is similar in size and shape to the archaebacterial proteasome. However, it is much more complex having seven different α-type and seven different β-type subunits arranged in four stacks in the order

$(\alpha_1...\alpha_7, \beta_1...\beta_7)_2$. The proteasomes from eukaryotes are even more complex. Their subunit pattern consists of multiple α and β type subunits related to either α or β subunits of the *Thermoplasma* proteasome (Tanaka, 1998).

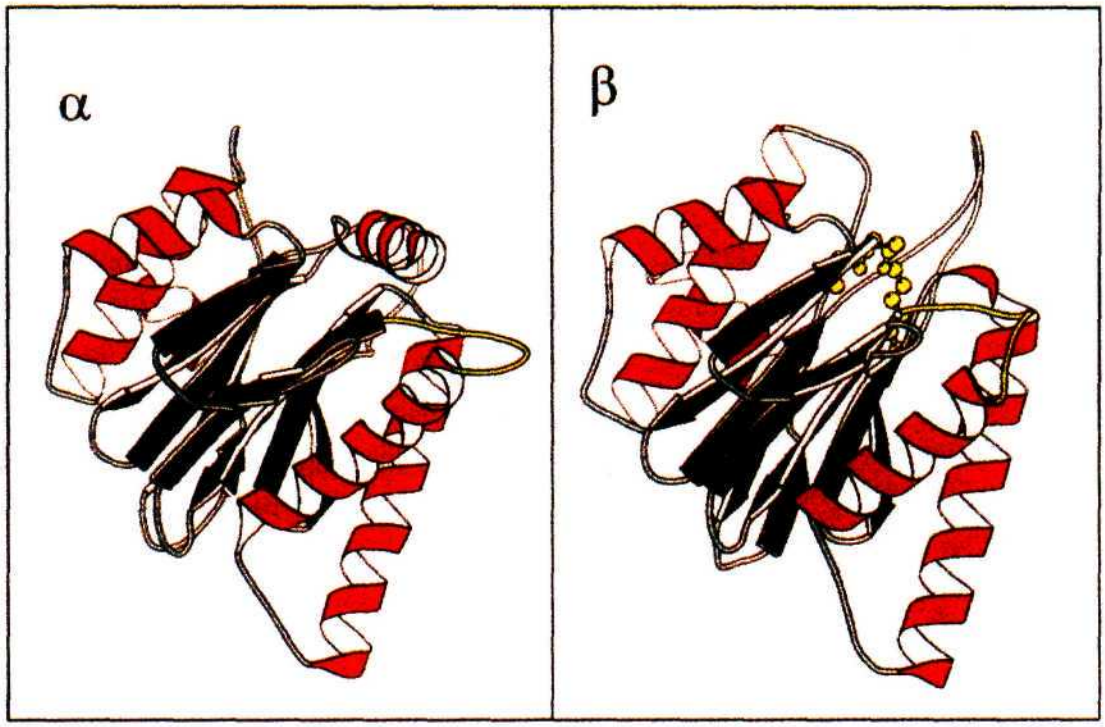

Figure 1.6. Structure of α- and β- subunits of *Thermoplasma acidophilum* proteasome.
The active site residues of the β- subunits Thr 1, Glu 17 and Lys 33, are shown in ball- and -stick (yellow) representation. Helices are shown in red, β-Strands and loops are blue (Baumeister *et al.* 1997).

The subunit compositions of the 20S proteasome of eukaryotic cells can vary in a given species and are subject to precise regulation. For example, γ-interferon treatment of cells induces the replacement of three β subunits by distinct, newly synthesised subunits, the LMP1, LMP2 and MECL1 subunits (Brown *et al.*, 1993; Akiyama *et al.*, 1994). These alter the activity of the proteasome to favour antigen presentation over other functions (Gaczynska *et al.*, 1993).

Nearly all α and β subunits contain several glycine residues and a common GXXXD motif. However, the amino acid sequences of α and β families differ in several respects. The α subunits are more conserved than the β, both within a given species and between species (Coux *et al.*, 1994). The main difference between α- and β-type subunits lies in a highly conserved N-terminal extension of the α-type subunits, part of which (residues 20-30)

forms an α-helix across the top of the central β sandwich (Fig. 1.6). All α subunits contain an RPXG motif. Several of the α subunits contain nuclear localization signals (NLS), as well as putative motifs that are complementary to the NLS (cNLS) that regulate nuclear localization (Nederlof *et al.*, 1995). Phosphorylation is thought to regulate proteasome translocation across the nuclear membrane by inducing conformational changes that modulate its binding to the NLS receptor on the nuclear pore (Castano *et al.*, 1996).

The α subunits by themselves have the capacity to form rings, but the β-subunits do not. Thus, the assembly of the α-rings is a prerequisite for the formation of the β-rings (Zwickl *et al.*, 1994). The α subunits constitute a physical barrier that limits the access of cytosolic proteins into the inner proteolytic chamber (Lowe *et al.*, 1995). The unfolded polypeptide substrates wind their way through the outer cavities (by unknown mechanism) to the proteolytic active site cleft in the central cavity. The polypeptides are then degraded to peptides of narrow size range (4 to 10 residues) (Wenzel *et al.*, 1994; Ehring *et al.*, 1996). In vertebrates, the focused product length of the proteasome has been exploited by the immune system for production of peptides displayed by the class 1 major histocompatibility complex (MHC I) (Rock *et al.*, 1994; Heemels and Ploegh, 1995).

1.7.5.2 Mechanism of action

The proteasome fold is prototypical of a new family of proteins called the N-terminal nucleophile (Ntn) hydrolases which have a unique, "single-residue" active site (Brannigan *et al.*, 1995; Duggleby *et al.*, 1995). In *Thermoplasma* proteasome, the single-residue is the N-terminal Thr of the β subunit which serves both as the catalytic nucleophile and the primary proton acceptor (Lowe *et al.*, 1995; Seemuller *et al.*, 1995). The nucleophilic attack is initiated when the free N-terminus (the primary proton acceptor) removes the proton from the catalytic residue's side chain (the nucleophile). This transfer, at least in the proteasome and penicillin cyclase, involves an intermediate water molecule (Fig. 1.7).

Three different N-terminal residues can act as the nucleophile in Ntn hydrolases: serine (penicillin acylase), cysteine (glutamine PRPP amidotransferase), or threonine (the proteasome) (Brannigan *et al.*, 1995; Lupas and Baumeister, 1998). In all Ntn hydrolases, the amide backbone group of a residue at the C-terminal end of Strand β4 (Gly 47 in *Thermoplasma* β subunit) is involved in forming the oxyanion hole. Besides the N-terminal threonine, the proteasome requires two further residues for activity (Seemuller *et*

al., 1995, 1996): a lysine residue (Lys 33 in *Thermoplasma* β subunit) and a glutamic acid residue (Glu 17 in *Thermoplasma* β subunit). The exact role of Lys 33 and Glu 17 is yet to be clarified. However, they form a salt bridge across the bottom of the active site. This may lower the pKa of the N-terminus by electrostatic effects, thus facilitating its function as a reversible proton acceptor, or it may actually participate in the delocalisation of the threonine side-chain proton by forming a charge relay system (Fig. 1.7B; Löwe *et al.*, 1995).

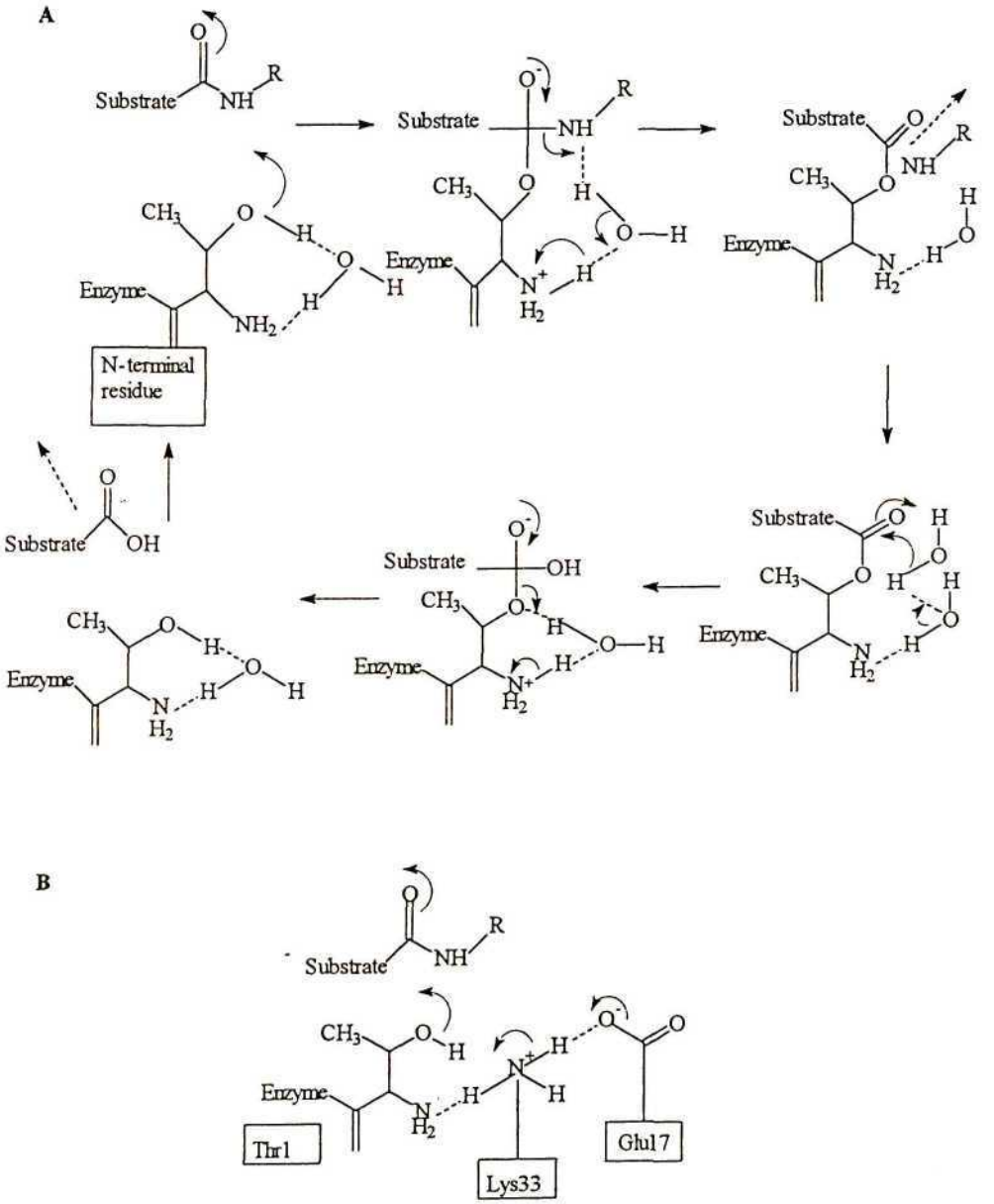

Figure 1.7. Putative catalytic mechanism of the 20S proteasome

Panel A shows the general mechanism of Ntn hydrolases and panel B the potential involvement of βLys33 and βGlu 17 as a charge relay system in the delocalization of the βThr1 side-chain proton (after Lupas and Baumeister, 1998)

A third residue (Asp 166 in *Thermoplasma* β subunit) may also be involved in formation of the active site, possibly via electrostatic effects, as its mutation in *Thermoplasma* inactivates the proteasome (Seemuller *et al.*, 1996)

1.7.5.3 26S Proteasome

The 26S proteasome is a very large protease complex with a molecular mass of approximately 2000 kDa (Koster *et al.*, 1995). It is formed by a central 20S core complex and two 19S cap complexes that associate with the two termini of the core in an energy dependent manner (Chu-Ping *et al.*, 1994). The PA 700 (or 19S cap) complexes are composed of 18 different subunits that range in molecular mass from 25 to 110 kDa. PA 700 is thought to function as the 'mouth' of the proteolytically active 20S core. It provides components necessary for the selective degradation of ubiquitinated proteins. These include binding sites for Ubiquitin (Ub)-chains (Deveraux *et al.*, 1994), isopeptidase activity and many ATPases, as well as the capacity to activate the peptidase activities of the 20S particle.

The structure of the 26S complex is shown in Figure 1.8. It is a labile structure that is stabilized *in vitro* by glycerol and ATP. In contrast to the 20S proteasome which degrades unfolded proteins in an energy-independent manner (Hilt and Wolf, 1996), degradation by the 26S complex is ATP-dependent and directed towards ubiquitinated and certain non-ubiquitinated proteins. The polypeptides comprising the 19S regulatory component of the 26S complex consist of ATPase and non-ATPase subunits. These ATPase subunits are thought to be responsible for the unfolding and transport of substrate proteins into the 20S core. The non-ATPase subunits do not belong to the ATPase family and lack the capacity to bind ATP (Dubiel *et al.*, 1992). These subunits are thought to serve diverse functions such as allowing association and activation of the 20S particle, capture of substrates, release and disassembly of the poly-ubiquitin chains, maintenance of the particle's structure, and delivery of the substrate into the 20S particle (Koster *et al.*, 1995; Coux *et al.*, 1996; Baumeister *et al.*, 1997, 1998).

In eukaryotic cells, degradation of many proteins proceeds via a polyubiquitination process. This involves an initial modification of the protein by conjugation to ubiquitin (Hochstrasser, 1996). This is a complex process involving the formation of a thioester bond at the C-terminus of ubiquitin. This reaction is catalysed by the ubiquitin activating

enzyme E1. Ubiquitin is then transferred to the active cysteine group of the soluble ubiquitin carrier protein E2. The E2 transfers the attached ubiquitin to the ε-amino groups of lysine residues of a protein substrate. This process in some cases needs cooperation with a ubiquitin protein ligase E3 (Hochstrasser, 1996). After its poly-ubiquitination, the protein substrates are recognized by the 26S proteasome and rapidly degraded into peptides.

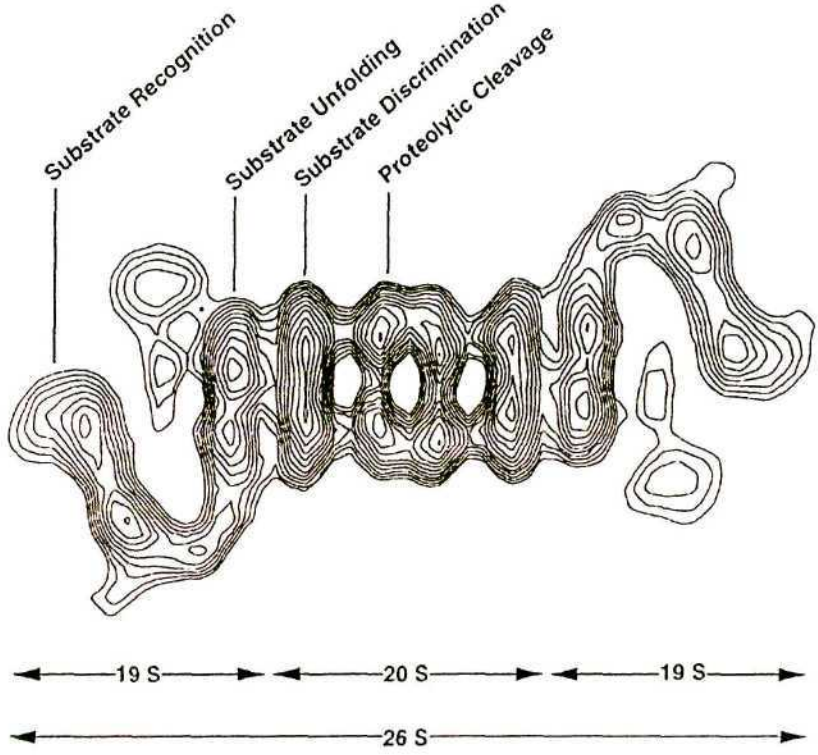

Figure 1.8. Contour plot of the 26S proteasome complex with putative functions assigned to parts of the complex (Koster *et al.*, 1995)

The eukaryotic proteasome was originally defined as a proteinase containing three active sites capable of cleaving peptide bonds after hydrophobic, basic and acidic amino acid residues. Thus the proteasome was considered to have chymotrypsin-like, trypsin-like and peptidylglutamyl peptide bond hydrolysing (PGPH) activities (Cardozo *et al.*, 1993). On the basis of studies with small synthetic peptides and protease inhibitors, as many as five distinct proteolytic activities have been assigned to the eukaryotic proteasome (Orlowski *et al.*, 1993). The newer activities were termed "branched chain amino acid preferring" or BrAAP and "small neutral amino acid preferring" or SnAAP. Pereira (1992a) also defined a distinct catalytic activity responsible for the initial degradation of β-casein, termed 'caseinolytic". These activities appear to be catalyzed by distinct active sites. Point mutations in β subunits, or changes in subunit composition (e.g. after γ-interferon

Point mutations in β subunits, or changes in subunit composition (e.g. after γ-interferon treatment) alter these activities differentially (Hilt and Wolf, 1995). These activities are inactivated by 3,4-dichloroisocoumarin (DCI) (Cardozo *et al.*, 1992) whereas the activities of BrAAP and SnAAP appear to be stimulated (Orlowski *et al.*, 1993). The proteasomes of *Thermoplasma* and *Rhodococcus* have only one major "chymotryptic"activity (Dahlmann *et al.*, 1992; Tamura *et al.*, 1995), in accordance with the fact that they have only one type of active site.

Three energy-independent large protease complexes have been described, which are traversed by a channel that widens into a large cavity. These are bleomycin hydrolase/Ga16 (Joshua-Tor *et al.*, 1995), the tricorn protease (Tamura *et al.*, 1996) and a giant protease (Geier *et al.*, 1999). Bleomycin hydrolase is a papain type cysteine protease. However, the active sites of the tricorn protease or the giant protease have yet to be defined.

1.7.5.4 Regulation of proteasome proteinase activities

Precise regulatory mechanisms must have evolved to allow selective protein breakdown in the cytosol and nucleus while preventing generalised proteolytic damage to the cell. Two structural features of the proteasome, the inclusion of its proteolytic sites within the internal chamber, and the restricted opening of the α-rings, reduce the chances of non-specific digestion of cell constituents (Baumeister *et al.*, 1998). In eukaryotes the requirement for ubiquitination of substrates and the machinery of the 19S complex probably evolved to ensure further selectivity. In addition, certain inherent regulatory properties and cellular inhibitory factors appear to help maintain the proteasome in an inactive form. The activity of the 20S proteasome is latent in freshly prepared extracts or when isolated by gentle methods. These proteasomes are inactive against protein substrates and degrade peptide substrates only slowly. Glycerol helps maintain the particle in a latent state (McGuire *et al.*, 1989) and presumably preserves the 20S and 26S complexes in their most physiological forms (Dahlmann *et al.*, 1992).

Dramatic stimulation of proteasome activities can be induced by various treatments of the crude proteasome containing extract, for example: by performing purification in the absence of glycerol, by incubation at 37°C, by heating at 55°C, by dialysis against water and by incubating with basic polypeptides, SDS, guanidine hydrochloride, or fatty acids

(Rivett, 1993). Most treatments that activate the 20S particle are known to disturb protein conformation. Thus, activation presumably involves either a conformational change in the β subunits that enhances catalytic activity, or an alteration in the α ring that facilitates substrate entry. Such modulatory effects may be mediated *in vivo* by specific endogenous activators or protein inhibitors. Several different endogenous inhibitors have been identified including a 200 kDa protein composed of identical 50 kDa subunits (Li and Etlinger, 1992); a 240 kDa protein (a hexamer of a 40 kDa subunit) (Guo *at al.*, 1994); and a 60 kDa inhibitor protein (a dimer of a 31 kDa subunit) (Chu-Ping *et al.*, 1992).

Protein complexes that associate with the eukaryotic 20S proteasome, and dramatically enhance its activity, have also been characterised. One is PA 700 (Chu-Ping *et al.*, 1994). This is the 19S complex that combines with the 20S proteasome in an ATP-dependent reaction to form the 26S complex. Another activator is PA28 (Chu-Ping *et al.*, 1992; Kuehn and Dahlmann, 1996). This is the 11S regulator that associates with the 20S proteasome in the absence of ATP (Kania *et al.*, 1996). Interestingly, a recent report indicates that the 20S proteasome can bind both PA700 and PA28 simultaneously to form an even larger ternary complex (Hendil *et al.*, 1998), but the significance of this is not clear.

Proteasome activities are inhibitable by specific peptide aldehydes. Thus, acetyl-Leu-Leu-norleucinal (also called calpain inhibitor 1) and chymostatin reversibly inhibit the chymotrypsin-like activity, while leupeptin inhibits the trypsin-like activity (Wilk and Pereira, 1993). Cbz-Leu-Leu-leucinal (MG132), is a potent inhibitor of the chymotryptic, postglutamyl, and BrAAP activities (Jensen *et al.*, 1995). Since MG132 readily enters cells (Reed *et al.*, 1995) it may prove to be useful for intracellular studies. A similar, but less potent inhibitor is Cbz-Leu-Leu-norvalinal (MG115) (Rock *et al.*, 1994). It may also prove to be a useful probe. A specific proteasome inhibitor that is not known to inhibit any other protease is the natural product lactacystin (Fenteany *et al.*, 1995). Lactacystin is converted in aqueous solution to lactone, which modifies N-terminal threonine residue involved in the catalysis by proteasomes (Dick *et al.*, 1996). The serine protease inhibitor DCI can also block multiple activities of the proteasome (Cardozo *et al.*, 1992) by reacting with the N-terminal threonine residues of the β subunits. However, DCI is much less specific and, under certain conditions, activates the proteasome (Cardozo *et al.*, 1992). Hence, it may not be useful for studies in intact cells.

Some of these agents block up to 90% of proteolysis in cultured mammalian cells, including the degradation of highly abnormal proteins, as well as the short- and long-lived normal proteins (Rock *et al.*, 1994). Thus, the proteasome may be implicated as the primary site for breakdown of most proteins in growing cells. However, other proteolytic systems (e.g. lysosomal proteolysis) can be important in non-growing cultures and in specialised cells (Gronostajski *et al.*, 1984; Scory *et al.* 1999).

1.7.5.5 Cellular functions of the proteasome

The primary function of the proteasome is to catalyze the breakdown of proteins into small peptides, most of which are then hydrolysed to amino acids. The proteasome may also be important in removal of abnormal proteins (Hilt and Wolf, 1995). The ubiquitin-proteasome pathway has been shown to catalyse the regulated proteolytic processing of a large, inactive precursor into an active protein, NF-κB (Palombella *et al.*, 1994). NF-κB is a transcriptional regulator for the excretion of various proteins critical in immune and inflammatory responses, including cytokines and adhesion molecules (Read *et al.*, 1995). One of its subunits, p50, is generated by proteolytic processing of an inactive 105 kDa precursor. The p50 is then maintained in the cytosol together with the p65 subunit in an inactive complex that is bound to an inhibitory protein 1κBα. Inflammatory signals (e.g. TNF-α) activate NF-κB by signalling the complete degradation of the inhibitor, 1kBα, and stimulating the proteolytic processing of the p105 precursor, catalysed by the 26S proteasome (Palombella *et al.*, 1994).

In higher vertebrates, the proteasome is thought to generate the peptides presented by MHC class I molecules of circulating lymphocytes (Rock *et al.*, 1994; Dick *et al.*, 1996). In this process, peptides generated during protein breakdown are transported into the endoplasmic reticulum via TAP1/TAP2 transporter. Here they are bound to MHC class I molecules before delivery to the cell surface. The presentation of these peptides enables the immune system to screen for and destroy, cells expressing viral or other unusual polypeptides. Indeed, the proteasome-specific inhibitor lactacystin blocks the generation and presentation of antigens by human and murine MHC class 1 molecules to cytotoxic T lymphocytes (Rock *et al.*, 1994, Cerundolo *et al.*, 1997).

The proteasome also play a key role in cell-cycle regulation. Eukaryotic cell division is controlled by cyclin-dependent protein kinases (CDKs). The appearance and disappearance of active kinase complexes during different phases of the cell cycle is regulated by synthesis and proteolytic degradation of specific cyclins (Yaglom *et al.*, 1995). There is evidence that the proteasome might be important for cell cycle regulation by degrading cyclins that act in different phases of the cell cycle (Glotzer *et al.*, 1991). Further, proteasome-mediated proteolysis has been implicated in apoptotic cell death (Drexler, 1997)

Finally, the proteasome also functions in the regulation of various metabolic adaptations. For example, the 26S complex degrades ornithine decarboxylase (ODC) in a ubiquitin-independent manner (Hershko and Ciechanover, 1998). This is a short-lived, rate-limiting enzyme in polyamine biosynthesis. Because polyamines are toxic in high levels, cells have evolved negative feedback mechanisms to inactivate and rapidly degrade ODC. Accumulation of polyamines induces the specific inhibitory protein, antizyme (AZ) (Hayashi and Murakami, 1995). AZ binds to ODC, inactivates it and triggers its degradation. When associated with AZ, ODC is rapidly hydrolyzed by the ATP-dependent 26S complex.

1.8 Objectives of the current study

Proteases play diverse functions in the parasite life cycle and pathology of parasitic infections (Lonsdale-Eccles, 1991; North, 1991). So far different classes of proteases have been identified and characterised in parasitic protozoa, including cysteine proteases from *T. brucei* (Mottram *et al.*, 1989; Troeberg *et al.*, 1996), *T. congolense* (Mbawa *et al.*, 1991b), *T. cruzi* (Engel *et al.*, 1998), a trypanosome-released cytosolic oligopeptidase (Morty *et al.*, 1998) and membrane-bound metalloproteinases of *T. brucei* (El-Sayed and Donelson, 1997) and *Leishmania* (Etges and Bouvier, 1993). Whereas MCP is ubiquitous and has been studied extensively in archaebacterium *Thermoplasma* and eukaryotic cells (reviewed by Coux *et al.*, 1996; Tanaka, 1998), there is a dearth of information on MCP from parasitic protozoa.

MCP is the major non-lysosomal proteolytic system that plays a central role in cytosolic and nuclear proteolysis (Section 1.7.5.5). The physiological role of the MCP has been shown to be necessary for cell viability. Considering that the MCP plays a central role in

key processes in the parasite, together with the established role of proteases in pathogenesis of parasitic diseases (Section 1.5), a better understanding of the structure and function of MCP may be essential for designing drugs or vaccines targeting the parasite MCP or MCP components.

The objectives of this study were to characterise a multicatalytic protease complex from *T. brucei* (MCP-Tb) and compare its biochemical and structural properties with the well-characterised MCP from bovine host (MCP-rbc). To achieve this, MCP-Tb and MCP-rbc were purified from *T. brucei* and bovine red blood cells respectively (Chapter 3). A number of the fundamental characteristics of the enzymes were investigated, including substrate specificity, inhibition and pH profiles (Chapter 3).

Once purified, structural studies on MCP-Tb and MCP-rbc were carried out by electron microscopy as described in Chapter 4. The N-terminal sequence of a subunit of MCP-Tb was determined for comparison with the primary structures of other proteasomes. The need to study the possible role of MCP-Tb in post-translational processing of VSG necessitated purification of VSG from *T. brucei*. Antibodies were raised against both VSG and MCP-Tb in chickens. The anti-MCP-Tb antibodies were used to assess antigenic similarities between the parasite MCP-Tb and the host MCP-rbc and to study the effect of the antibody on the catalytic activity of MCP-Tb (Chapter 4).

MCP-Tb was localised in the parasite using biochemical and immunocytochemical methods (Chapter 5). The antibodies raised against MCP-Tb and VSG were used for the immunolocalisation studies. Considering that trypanosome proteases may be actively or constitutively secreted, or simply released into the host bloodstream upon complement mediated lysis, the possible interaction between the parasite MCP-Tb and the host regulatory molecule, proteasome activator PA700 was investigated. Proteasome activator PA700 was purified from bovine red blood cells and its effect on MCP-Tb activity was tested *in vitro* (Chapter 6).

Chapter 2

General materials and methods

2.1 Introduction

The experimental techniques used in this study mostly involved protein purification and characterisation, and immunochemical protocols. For convenience, this chapter presents the basic techniques common to the different areas covered in this study. In order to maintain the logical sequence of information presented, those techniques pertaining to specific areas of study will be described in the relevant chapters.

2.2 Materials

Common chemicals used in this study were from Sigma, BDH or Boehringer Mannheim, and were of analytical grade or purer. Lowicryl K4M resin was from Electron Microscopy Sciences, Fort Washington, PA, USA. DEAE-cellulose (DE-53) was from Whatman Biochemical Systems, Maidstone, U.K. Sephacryl S-300 and molecular mass markers were from Pharmacia, Uppsala, Sweden. Ampholytes (Servalyt), Serva Blue G, acrylamide, and N,N,N',N'-tetramethyl-ethylenediamine (TEMED) were from Serva, Heidelberg, Germany. Nitrocellulose transfer membrane for Western blot was from Micron Separations Inc., MA, USA. Immobilon-P^{SQ} transfer (PVDF) membrane for microsequencing was from Millipore Corporation, Bedford, MA, USA. Percoll®, HA-Ultrogel, Q-sepharose, protease inhibitors, Leu-Leu-Glu-βNA, hypoxanthine, sodium orthovanadate, ouabain, β-casein, rabbit anti-chicken IgG-alkaline phosphatase conjugate, and Sigma Fast® 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate were purchased from Sigma Chemicals Co., St. Louis, MO, USA. All other peptide substrates used were from Bachem Feinchemikalien, Bubendorf, Switzerland. Horseradish peroxidase (HPRO) was from Seravac South Africa. Freund's complete and incomplete adjuvants were purchased from Gibco Laboratories, Life Technologies, Inc., OH, USA. 2,2 Azino-di-[3-ethylbenzthiazoline sulphonate (6)] (ABTS) was from Boehringer Mannheim, SA. Microtitre plates Nunc-Immuno Plate MaxiSorp™ used in enzyme-linked immunosorbent assays (ELISA) and FluoroNunc™ MaxiSorp plates used for fluorescence assays were from AEC-Amersham, South Africa.

2.3 Parasite Isolation

Trypanosoma brucei brucei clone ILTat 1.1 (originally derived from East African Trypanosomiasis Research Organization, stock EATRO 795) was used in this study. Bloodstream forms were grown in 4-month-old Sprague Dawley rats. Trypanosomes were separated from blood cells and platelets by passing infected blood from infected rats through the anion exchanger DEAE-cellulose (Lanham and Godfrey, 1970). Separation depends on the difference between the surface charge of blood cells and that of parasites. The more negatively charged blood components adsorb to the DEAE-cellulose while the less negatively charged parasites are eluted.

Although surface charge differs between species of salivarian trypanosomes (Lanham and Godfrey, 1970) it is usually sufficiently different from that on the rodent blood cells and platelets for successful separation under standard conditions of pH and ionic strength. However, the negative charge density on the erythrocytes also varies with mammalian species. Those with the lowest level of charges may be eluted from DEAE-cellulose under the standard buffer conditions, so that separation from trypanosomes may not occur. Although such blood cells could be adsorbed, by lowering the ionic strength of the buffer, too great a reduction may cause adsorption of the infecting trypanosomes. The ionic strength of the phosphate saline glucose (PSG) recommended for the isolation of trypanosomes from rat blood is 0.217. In each case, separations were carried out at pH 8.0 (Lanham and Godfrey, 1970).

2.3.1 Reagents

Percoll solution [2% (m/v) glucose, 250 mM sucrose, 100% (v/v) Percoll®, pH 7.4]. Glucose (2.0 g) and sucrose (8.55 g) were dissolved in Percoll® (100 ml) and adjusted to pH 7.4 by addition of solid HEPES.

Phosphate saline glucose (PSG), pH 8.0. Na_2HPO_4 (8.088 g), $NaH_2PO_4.2H_2O$ (0.467 g), NaCl (2.5 g), and glucose (10.0 g) were dissolved in 950 ml of dist. H_2O, adjusted to pH 8.0 and made up to 1 litre.

Sodium citrate -PSG [0.5% (m/v) Na-citrate]. Sodium citrate (0.5 g) was dissolved in 100 ml PSG 6:4, pH 8.0.

2.3.2 Procedure

The blood parasites were isolated on the fifth day after inoculation (at peak parasitaemia). Rats were anaesthetised using chloroform, blood was obtained by cardiac puncture and collected into 0.5% citrate (0.5 ml). The samples were mixed and kept on ice. Trypanosomes were separated from blood cells by a combination of centrifugation on isopycnic Percoll® gradients (Grab and Bwayo, 1982) and chromatography on DEAE-cellulose (Lanham and Godfrey, 1970). Blood was mixed with Percoll® in the ratio 1:1 (v/v) and centrifuged (17 000 x g, 15 min, 4°C). After centrifugation, trypanosomes floated as a single band on top of the Percoll®-sucrose-glucose gradient, while platelets were found in the middle layer and red blood cells pelleted at the bottom of the centrifuge tube. The band of trypanosomes was removed by aspiration and mixed with 3 parts PSG, pH 8.0 containing 0.1 mM hypoxanthine (PSGH) (Lonsdale-Eccles and Grab, 1987) and centrifuged in a microfuge (421 x g, 15 min; 25°C). Pelleted trypanosomes were resuspended in a minimum volume of PSG and the pH adjusted to 8.0 by addition of solid Tris base. The parasite preparation was then layered on DEAE-cellulose exchange resin equilibrated in PSGH, pH 8.0 in a Büchner funnel.

Parasites were eluted from the column using PSG into a beaker containing 200 ml PSGH, pH 7.4. The cells were pelleted, washed three times in PSGH buffer, pH 7.4 (1 000 x g, 15 min, 4°C) and resuspended in the same buffer. Cell numbers were determined in a hemocytometer when necessary. Pelleted cells were usually frozen and stored at –80°C for subsequent enzyme purification. No appreciable loss of enzyme activity was observed.

2.4 Protein assay

The Bradford dye-binding assay (Bradford, 1976) as modified by Read and Northcote (1981) was routinely used for protein determination, since it is a rapid and very sensitive protein quantitation technique. The method is unaffected by most laboratory reagents except high concentrations of detergents (e.g. 1% Triton X-100). Under acidic conditions, the Coomassie Blue G-250 dye (Bradford reagent) specifically binds to Arg and Phe residues in protein molecules, forming a blue complex that can be monitored at 595 nm (Bradford, 1976). The assay used in this study followed the original Bradford method, but using Serva blue G dye (Read and Northcote, 1981). The modified assay, minimizes the protein-to-protein variability

in dye binding and increases the sensitivity of the assay (Read and Northcote, 1981). This micro-assay method was used to quantify protein in the 1-5 μg range.

2.4.1 Reagents

Dye Reagent. Serva blue G dye (50 mg) was dissolved in 88% (v/v) phosphoric acid (50 ml) and 99.5% (v/v) ethanol (23.5 ml). The solution was made up to 500 ml with dist.H_2O and stirred for 30 min on a magnetic stirrer. The resulting solution was filtered through Whatman No.1 filter paper and stored in an amber coloured bottle. The solution could be stored for up to 6 months. Visual checks were made before use and if precipitation was visible, the reagent was filtered and re-calibrated.

Micro-assay. Standard protein (0-50 μl of a 0.1 mg/ml standard ovalbumin solution) or sample was diluted to a final volume of 50 μl with dist.H_2O, or buffer, in 1.5 ml microfuge tubes. Dye reagent (950 μl) was added, and the content of the tube mixed by inversion of the tube. The colour was allowed to develop for 2 min after mixing and the absorbance read at 595 nm. Assays for construction of a standard curve were carried out in triplicate at 5 concentrations of ovalbumin. Results were calculated by linear regression analysis of the standard curves, for each assay type and for every batch of dye reagent made up.

2.5 Concentration of Samples

It was often necessary to concentrate protein samples (e.g. before proceeding to the subsequent step of purification, or for gel electrophoretic analyses, or for inoculation into experimental animals for antibody production). The use of commercial polyethylene glycol (PEG) was found to be a simple and efficient method of concentrating protein samples. The high M_r (20 000) form of PEG was used so that it was excluded by M_r 12 000 cut-off dialysis tubing. Concentration by dry, highly soluble polymers such as PEG or sucrose is based on the principle of movement of water along a concentration gradient out of the dialysis bag to dissolve the dry polymer. Dialysis against sucrose was not desirable because of the presence of variable amounts of sucrose in the concentrated protein sample which diffuse into the dialysis bags. Ultrafiltration on the other hand has been found to denature certain proteinases such as rabbit cathepsin L (Mason *et al.*, 1984). Further, high glycerol requirement (20%) by MCP-Tb in the buffers made the samples too viscous to concentrate in good time by ultrafiltration.

2.5.1 Dialysis against PEG

When a protein solution needed to be concentrated, the sample was sealed in a dialysis bag (M_r 12 000 cut-off limit), which was placed onto a thick layer of PEG (M_r 20 000) in a plastic tray at 4^0C. Once the sample was sufficiently concentrated (5-10 fold), the bag was briefly rinsed in dist.H_2O (to remove adhering PEG). The bag was then cut open and the sample squeezed out. Rubber gloves were worn at all times during this procedure to prevent contamination of the protein samples (e.g. by skin cells).

2.6 Polyacrylamide gel electrophoresis

Gradient polyacrylamide gel electrophoresis under non-denaturing conditions was used to assess the homogeneity of the purified multimeric protein samples. The molecular mass of the native protein was estimated by comparison with high molecular mass markers separated on the same gradient gel as the protein of interest (Laemmli, 1970). This technique was used together with western blotting (Section 2.11.) to identify proteins, and to assess the specificity of antibodies raised against the purified proteins. Because many proteases retain their catalytic properties after electrophoresis on non-denaturing polyacrylamide gels, the activity of such proteases can be detected as the clear sites remaining from digestion of gelatin or fibrinogen that has been copolymerized with the polyacrylamide gel (Heussen and Dowdle, 1980; Lonsdale-Eccles and Mpimbaza, 1986). In the present study, zymogram profiles were obtained on gelatin-containing polyacrylamide gels (Section 2.7).

The purified multicatalytic protease (Section 3.2) was found to be susceptible to denaturation by anionic detergents such as SDS. SDS-PAGE under either reducing or non-reducing conditions was thus used only for the characterisation of the purified protein, especially the determination of the relative molecular mass value of its subunits. Ideally, SDS interacts with most proteins producing negatively charged rod-like SDS-protein complexes. The length of these complexes corresponds to the molecular mass of the protein. If additionally the protein is treated with a reducing agent, such as 2-mercaptoethanol, the protein will be reduced to its constituent subunits, resulting in improved binding of SDS, giving a more accurate estimation of molecular mass. The negatively charged protein-detergent complexes with similar charge-to-mass ratios migrate anodally in an electrical field. The inverse relationship between the

distance migrated in a gel by a protein and the logarithm of its molecular mass, allows the construction of a standard curve for M_r determination. This is done by running standard proteins of known M_r alongside the proteins to be characterised.

2.6.1 Reagents

Solution A :Monomer Solution [30% (m/v) acrylamide, 2.7% (m/v) N,N' methylene bisacrylamide]. Acrylamide (73 g) and N,N' methylene bisacrylamide (2 g) were dissolved and made up to 250 ml with dist. H_2O and stored in an amber coloured bottle at 4°C.

Solution B :4 x Running Gel Buffer (1.5 M Tris-HCl, pH 8.8). Tris (18.16 g) was dissolved in approximately 70 ml of dist. H_2O, adjusted to pH 8.8 with HCl and made up to 100 ml.

Solution C :4 x Stacking Gel Buffer (0.5 M Tris- HCl, pH 6.8). Tris (6.06 g) was dissolved in 70 ml dist. H_2O, adjusted to pH 6.8 with HCl and made up to 100 ml. This buffer was made freshly each week. This was because of its poor buffering capacity at 2.1 pH units below the buffer's pKa at 4°C, which results in pH drift and anomalous running patterns in non-reducing polyacrylamide gel electrophoresis.

Solutions A, B, and C were filtered through Whatman No.1 filter paper before storage.

Solution D :10% (m/v) SDS. SDS (10.0 g) was dissolved in 100 ml dist. H_2O with gentle heating if necessary.

Solution E :Initiator [10% (m/v) ammonium persulfate (APS)]. Ammonium persulfate (0.05 g) was made up to 0.5 ml just before use.

Solution F :Tank Buffer [25 mM Tris-HCl, 192 mM glycine, 0.1% (m/v) SDS, pH 8.8]. Tris (12.0 g) and glycine (57.6 g) were dissolved and made up to 4 litres with dist. H_2O. For denaturing SDS-PAGE, 40 ml of 10% SDS Stock (Solution D) was added to 4 litres of Solution F for use in the Biorad Protean II xi Electrophoresis Cell.

Solution G :Reducing Treatment Buffer [125 mM Tris- HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4.0 ml) (Solution D), glycerol (2.0 ml) and 2-mercaptoethanol (1.0 ml) were made up to 10 ml with dist. H_2O.

Solution H :Non-reducing Treatment Buffer [125 mM Tris-HCl, 4% (m/v) SDS 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4.0 ml) (Solution D) and glycerol (2.0 ml) were made up to 10 ml with dist. H_2O.

Solution I :Non-denaturing Treatment Buffer [125 mM Tris-HCl, 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml) and glycerol (2.0 ml) was made up to 10 ml with dist. H_2O.

Stain Stock Solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1.0 g) was dissolved in 100 ml of dist. H_2O mixed by magnetic stirring for 1 h at room temperature. The solution was filtered through Whatman No.1 filter paper.

Staining Solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml) and made up to 500 ml with dist. H_2O.

Destaining Solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) was mixed with acetic acid (100 ml) and made up to 1 litre with dist. H_2O.

Destaining Solution II [7% (v/v) acetic acid, 5% (v/v) methanol]. Acetic acid (70 ml) was mixed with methanol (50 ml) and made up to 1 litre with dist. H_2O.

2.6.2 Procedure

Non-denaturing PAGE was performed in a Biorad Protean II Slab Cell assembled as described in the manufacturer's instruction manual. The mixed running gel solution (Table 1.1) was carefully poured into the space between the plates to a depth 1-2 cm from the top of the glass plate and overlayered with dist. H_2O to prevent inhibition of polymerization by oxygen. Once the gel had polymerized, usually about 45 min to 1 h, the water was removed with a syringe. Stacking gel monomer solution was poured in to cover a 10 well comb inserted to form the sample application wells. Once this gel polymerized (about 45 min) the comb was removed, and the wells rinsed with sample treatment buffer.

Polyacrylamide gradients (4.5-10%) were made with an external gradient former and introduced into the Protean II xi gel-sandwich assembly using a peristaltic pump.

Table 1.1. Formulation for running and stacking gels of different acrylamide concentrations

Reagent	Volume (ml)				
	Running gel (%)			Stacking gel (%)[a]	
	12.5	10.0	3.0	4.0	3.0
A	6.25	5.0	1.5	0.94	0.71
B	3.75	3.75	3.75	0.0	0.0
C	0.0	0.0	0.0	1.75	1.75
D	0.15	0.0	0.0	0.07	0.0
E	0.075	0.075	0.075	0.035	0.035
Dist.H_2O	4.75	6.15	9.65	4.30	4.83
TEMED	0.0075	0.0075	0.0075	0.012	0.012

[a]The (3.0%) stacking gel was used in non-denaturing 4.5-10% gradient gels, whereas the 4.0% stacking gel was used in combination with 12.5% running gels in non-denaturing and reducing SDS-PAGE.

The upper and lower electrode compartments were filled with Tank buffer (Solution F). Samples for non-reducing SDS-PAGE were combined with half their volume of non-reducing treatment buffer (Solution H) before loading onto the gel. For reducing SDS-PAGE, samples were combined with an equal volume of reducing treatment buffer (Solution G) and incubated in a boiling water bath for 2 min before loading. Non-reducing samples were also subjected to boiling except when enzyme activity needed to be retained in substrate gels (Section 2.7). A marker dye, bromophenol blue, which migrates with the buffer front, was added to each sample before loading into the gels. Suitable amounts of samples (about 3 µg) were pipetted into the wells. The gels were run at 40 mA per slab gel (Protean II xi apparatus) or 18 mA per mini gel (in Biorad mini gel apparatus) until the bromophenol blue tracker dye was about 0.5 cm from the bottom of the running gel. The gel was removed and placed into staining solution for 4 h. The gel was rinsed with dist. H_2O and placed in destaining solution I overnight, followed by soaking in destaining solution II to effect complete destaining. The gels were stored in polythene zip-seal bags and kept hydrated until photographed.

2.6.3 Silver Staining

Protein bands on polyacrylamide gels were silver-stained when a high degree of sensitivity was necessary for their detection. Silver staining is a method based on the reduction of silver ions to metallic silver and is capable of detecting as little as 100 ng protein per band (Blum *et al.*, 1987). The method of Blum *et al.* (1987) differs from previous methods in that it incorporates thiosulfate that not only increases sensitivity, but also complexes insoluble silver salts from the surface of the gels, resulting in clear background.

2.6.3.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) 37% (v/v) formaldehyde]. Methanol (50 ml), acetic acid (12 ml) and 37% (v/v) formaldehyde (50 µl) were made up to 100 ml with dist. H_2O.

Thiosulfate solution [0.02% (m/v) sodium thiosulfate]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in dist.H_2O (200 ml).

Silver nitrate solution [0.2% (m/v) $AgNO_3$, 0.075% (v/v) 37% (v/v) formaldehyde]. $AgNO_3$ (200 mg) was dissolved in 80 ml dist.H2O, 37% (v/v) formaldehyde (75 µl) added and volume adjusted to 100 ml with dist.H_2O.

Developing solution [6% (m/v) Na_2CO_3, 0.05% (v/v) 37% (v/v) formaldehyde, 0.0004% (m/v) $Na_2S_2O_3$]. Na_2CO_3 (6 g) was dissolved in 80 ml of dist.H_2O, 37% (v/v) formaldehyde (50 µl) and $Na_2S_2O_3.5H_2O$ (2 ml of thiosulfate solution above) added and the volume adjusted to 100 ml with dist.H_2O.

Stopping solution [50% (v/v) methanol, 12 % (v/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were diluted to 100 ml with dist.H_2O.

2.6.3.2 Procedure

Polyacrylamide gels, fixed overnight in fixing solution, were washed in 50% (v/v) ethanol (3 x 20 min), pre-treated in thiosulfate solution for 1 min and rinsed in dist.H_2O (3 x 20 sec). Following impregnation for 20 min in the silver nitrate solution, gels were rinsed in dist.H_2O (2 x 20 sec) and immersed in developing solution until bands were visible. The

gels were rinsed in dist.H_2O (2 x 2 min), immersed in stopping solution for 10 min and finally washed for at least 20 min in 50% (v/v) methanol.

2.7 Gelatin-Substrate Polyacrylamide Gel Electrophoresis

Zymogram analysis of proteinases was performed on non-denaturing polyacrylamide gels in which gelatin had been copolymerised with the acrylamide (Heussen and Dowdle, 1980). After electrophoresis, the gels were incubated in assay buffer containing appropriate cofactors to ensure specific enzyme activity. The presence of proteinase activity is indicated by clear bands against a stained gelatin background. In cases where SDS is used, the proteinases are renatured after electrophoresis but prior to incubation in the assay buffer. This is done by removal of SDS by incubation of the gel in 2.5% (v/v) Triton X-100 (Heussen and Dowdle, 1980) prior to incubation in the assay buffer.

2.7.1 Reagents

1% (m/v) gelatin in running gel buffer. Gelatin (0.1 g) was dissolved in running gel buffer (10 ml Solution B, Section 2.6.1) with gentle heating.

Overlay solution for substrate gels [0.05% (m/v) gelatin]. Gelatin (0.005 g) was dissolved in running gel buffer (2 ml, Section 2.6.1) with gentle heating and diluted to 10 ml with dist. H_2O.

2.5% (v/v) Triton X-100. Triton X-100 (5 ml) was diluted to 200 ml with dist. H_2O.

0.1% (m/v) amido black. Amido black (0.1 g) was dissolved in methanol:acetic acid:dist. H_2O in the proportions 30:10:60 (100 ml), and filtered through Whatman No. 1 filter paper before use.

2.7.2 Procedure

The procedure for gelatin-containing polyacrylamide gel electrophoresis was essentially the same as that described in Section 2.6.2 except that 0.1% (m/v) gelatin was copolymerized with the polyacrylamide (Heussen and Dowdle 1980). This was carried out by adding 1% (m/v) gelatin (1.5 ml) to running gel buffer (2.75 ml), Section 2.6.1. The gradient gels were cast as described in Section 2.6.2.

After electrophoresis, the gels containing SDS were incubated in two changes of 2.5% (v/v) Triton X-100 for 30 min (mini-gel) or 60 min (standard gels). The gel was then incubated in assay buffer for 4 h at 37°C. The gel was stained in 0.1% (m/v) amido black solution for 1 h, and destained in several changes of methanol:acetic acid:dist. H_2O (30:10:60). Proteolytic activity was observed as clear bands of gelatin digestion.

2.8 Two–Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional gel electrophoresis (2D-PAGE) separates proteins by their iso-electric points (pI) and electrophoretic mobilities (O'Farrell, 1975). In the first dimension, a sample is applied to an iso-electric focusing (IEF) acrylamide gel. Within this, the constituent proteins migrate through a pH gradient until they reach their respective pI. A protein's pI is the pH value where it has no net charge. Proteins in a sample thus become focused at their individual pI.

The second dimension further separates the proteins on the basis of their molecular sizes by using SDS-PAGE (Section 2.6). In the present study, 2D-PAGE was used to analyse the protein components of MCP-Tb and MCP-rbc.

2.8.1 First Dimension: IEF

2.8.1.1 Reagents

10% (v/v) Triton X-100. Triton X-100 (1 ml) was mixed with 9 ml of dd.H_2O.

Gel overlay (8 M Urea). Ultrapure urea (2.4 g) was dissolved in a total of 5 ml dd.H_2O. Aliquots (1 ml) were stored at -20°C.

Sample overlay [9.5 M urea, 0.5% (v/v) pH 3.5-10 ampholytes, 2% (v/v) pH 5-7 ampholytes]. Ultrapure urea (3.75 g), Pre-blended pH 3.5-10 ampholyte (Pharmacia) (125 µl) were dissolved in 2.5 ml of dd.H_2O and made up to 5 ml with dd.H_2O.

IEF sample buffer [9.5 M urea, 100 mM dithiothreitol, 4% (v/v) Triton X-100, 0.4% (v/v) pH 3.5-10 ampholyte, 1.6% (v/v) pH 5-7 ampholyte]. Ultrapure urea (0.57 g), dithiothreitol (15 mg), 10% (v/v) Triton X-100 (0.4 ml), Pre-blended pH 3.5-10 ampholyte (Pharmacia) (50 µl) were dissolved in a total of 1 ml of dd.H_2O.

IEF stock acrylamide [42.3% (T) acrylamide, 5.4% (C) N,N' methylene bisacrylamide]. Acrylamide (2.838 g) and N,N' methylene bisacrylamide (0.162 g) were dissolved in a total of 7.1 ml dd.H_2O. Stock could be stored for one month at -4°C.

IEF gel solution [8 M urea, 4.7% (T) acrylamide, 0.6% (C) N,N' methylene bisacrylamide, 1.7% (v/v) Triton X-100, 4% pH 5-7 ampholyte, 0.3% pH 3.5-10 ampholyte]. Ultrapure urea (3.45 g), IEF stock acrylamide (0.8 ml), 10% Triton X-100 (1.2 ml), Pre-blended pH 3.5-10 ampholyte (Pharmacia) (300 µl) were added and mixed in a total volume of 7.2 ml dd.H_2O. Aliquots (2.4 ml) were kept frozen at –80°C.

Equilibrating buffer [50 mM Tris-HCl, 2% (m/v) SDS, 50 mM dithiothreitol, 10% (v/v) glycerol, pH6.8]. Tris (0.061 g), 10% SDS (2 ml), DTT (0.076 g), glycerol (1 ml) were dissolved in 5 ml of dd.H_2O, adjusted to pH 6.8 with HCl, and made up to 10 ml with dd.H_2O.

2.8.1.2 Procedure

The first dimensional separation (IEF) was performed essentially according to O'Farrell (1975) in an IsoDalt 2-D electrophoresis apparatus (Hoeffer, San Fransisco, CA, USA). Polyacrylamide gels (4.7%T) were cast in glass tubes (16 cm x 1.5 mm in diameter). The tubes were blocked at one end with two layers of Parafilm®. Aliquots of IEF gel solution were thawed, brought to 25°C and degassed for 15 min. Fresh 10% ammonium persulfate (Section 2.6) (1.5 µl) and TEMED (1.05 µl) were added to the IEF gel solution (2.4 ml), swirled to mix, and slowly injected into the bottom of the tube to dislodge any air bubbles. After polymerisation (for at least 2 h), the tops of the gels were thoroughly rinsed with dd.H_2O. The Parafilm® was removed from the bottom of each tube, the tubes placed back in the casting stand, and gel overlay buffer applied to a height of 1 cm above the gel surface.

Solid urea was added to the sample, dissolved by flicking the tube until the sample was saturated. IEF sample buffer (10 µl) was added to the sample (50 µl) in a microfuge tube, mixed and centrifuged (15 000 x g, 30 sec) to remove undissolved materials. The tube gels were put into position in the assembled electrophoresis apparatus. The surfaces of the tubes were filled with catholyte (20 mM NaOH) solution and the lower buffer chamber

with anolyte (1M ortho-phosphoric acid). The core was carefully placed in the tank preventing air bubbles from accumulating at the bottom of the gels. Following prefocusing (15 min at 200 V, 30 min at 400 V, and 30 min at 600 V), samples (10-15 μg) were loaded at the catholyte end of the gel and overlaid with sample overlay solution using a syringe and needle. For good electrical contact, care was taken not to trap air bubbles in the tube during loading. The gel was run at 600 V for 13 h followed by 1000 V for 1 h. After electrophoresis, both the top and bottom of each gel was rinsed with dd.H_2O to remove residual base and acid on the gel that would interfere with measurement of the pH gradient. The tube gels were extruded using water pressure onto glass storage tubes containing equilibrating buffer (3 ml). The gels were either kept frozen at -80°C or embedded on the slab gel as quickly as possible to prevent diffusion of proteins from the gel.

2.8.2 Second Dimension: SDS-PAGE

The second dimensional separation was performed using Tricine-SDS-PAGE (Shägger and Von Jagow, 1987) to separate the subunits. The tube gels were equilibrated in gel buffer prior to second dimension electrophoresis.

2.8.2.1 Reagents

Agarose overlay [0.2% (m/v) agarose]. Agarose (0.1 g) was dissolved in 50 ml of equilibrating buffer (Section 2.8.1.1).

Anode buffer (0.2 M Tris-HCl, pH 8.9). Tris (96.88 g) was dissolved in approximately 3800 ml of dist. H_2O, adjusted to pH 8.9 with HCl and made up to 4 litres with dist. H_2O.

Cathode buffer [0.1 M Tris, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.2 g), Tricine (17.9 g) and 10% (m/v) SDS (10 ml) were dissolved and made up to1 litre with dist. H_2O.

Gel buffer [3.0 M Tris, 0.3% (w/v) SDS, pH 8.45]. Tris (72.7 g) and SDS (0.6 g) were dissolved in 150 ml of dist.H_2O, adjusted to pH 8.45 with HCl and made up to 200 ml with dist. H_2O.

Monomer solution [49.5% (w/v) Acrylamide, N,N' methylene bisacrylamide]. Acrylamide (48 g) and N,N' methylene bisacrylamide (3 g) were dissolved in a total of 100 ml of dist.H_20.

2.8.2.2 Procedure

A running gel was prepared by mixing monomer solution (32 ml), gel buffer (40 ml) and dist. H_2O (40 ml) and polymerisation initiated by addition of 10% (m/v) ammonium persulfate (0.4 ml) and TEMED (40 µl) (Section 2.6.1). The running gel mixture was poured between glass plates on assembled Biorad Protean II. Once the pair of slab gels (16 cm x 16 cm) had polymerised, a stacking gel, with a 2D-comb (1.5 cm) in place, was poured to the top of front glass plate. The stacking gel consisted of monomer solution (0.5 ml), gel buffer (1.5 ml), dd.H_2O (4 ml), 10% (m/v) ammonium persulfate (30 µl) and TEMED (12 µl). After polymerisation, the wells in the stacking gel were rinsed with dd.H_2O followed by gel buffer. A freshly thawed IEF tube gel was placed on a 16 cm longitudinally folded piece of Parafilm®. Excess equilibrating buffer was removed, and the gel was directed from the Parafilm® to the bevel at the top of the inner glass plate, starting at one side and proceeding across the gel. Care was taken that the tube gel was in contact with the slab gel over its entire length. All air bubbles were removed between the tube gel and the slab gel. An agarose overlay was added to ensure good contact between the gels and to prevent the tube gel from slipping off the slab gel. Low molecular mass markers were loaded in an adjacent well in the stacking gel.

The lower electrophoresis chamber was filled with anode buffer and the top chamber with cathode buffer. Electrophoresis was conducted at 80 V until the bromophenol blue marker dye entered the separating gel and at 100 V for the remainder of the electrophoretic run. At the end of the electrophoresis, one 2D-gel was silver stained (Blum *et al.*, 1987; Section 2.7) and the other proteins electrotransferred to polyvinylidene difluoride (PVDF) membranes (Section 2.9) for N-terminal sequencing of the protein component of interest.

2.9 Blotting of proteins onto PVDF membranes

Protein components of MCP-Tb separated by 2D-PAGE were electroblotted onto a PVDF membrane, essentially as described by Matsudaira (1987). The membranes are mechanically strong solid phase supports that bind proteins hydrophobically and have been used in immunoblotting applications as a substitute for nitrocellulose filters (see Section 2.11).

Because PVDF membranes are inert to most solvents (e.g. acetonitrile, trifluoroacetic acid, ethyl acetate, trimethylamine, and hexamine), they are ideal for use in automated gas-phase sequenators as a solid-phase support for sequence analysis.

2.9.1 Reagents

Electrotransfer buffer [10 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 10% (v/v) methanol, 0.1 mM thioglycollic acid, pH 11.0]. CAPS (5.533 g), methanol (250 ml) and thioglycollic acid (ρ = 1.33 g/ml; 17.5 µl) were dissolved in 2 litres of dd.H_2O, adjusted to pH 11.0 with NaOH and made up to 2.5 litres with dd.H_2O.

Stain solution [0.1% Coomassie Brilliant Blue R-250 in 50% (v/v) methanol]. Coomassie Brilliant Blue R-250 (0.1 g) was dissolved in methanol (50 ml) and the volume adjusted to 100 ml with dd.H_2O.

Destain solution [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (10 ml) were diluted to 100 ml with dd.H_2O.

2.9.2 Procedure

Following 2D-PAGE, the gels were soaked in CAPS buffer for 5 min to reduce the amounts of Tris and Tricine, PVDF membranes were pre-treated in 100% (v/v) methanol (15 s), dd.H_2O (5 min) and stored in electrotransfer buffer. The gel was sandwiched between a sheet of PVDF membrane and several sheets of filter paper and proteins to be electrotransferred (as per Section 2.11.2). Following transfer (200 mA, 2 h), the PVDF membrane was washed in deionised H_2O (2 min), immersed in stain solution (5 min), followed by destain solution (2 x 5 min). The membrane was finally rinsed in dd.H_2O (2 x 5 min), air dried and transferred to a zip-seal plastic bag for storage at –20°C until N-terminal sequencing was conducted.

2.10 Antibody production and isolation

Polyclonal antibodies used in immunocytochemistry (Section 5.5.2) and western blotting (Section 4.7) were raised in chickens by inoculation of 50 µg of protein (e.g. proteasome or VSG purified from *T. brucei*) intramuscularly into the breast muscle. The protein antigens were mixed with an equal volume of Freund's complete adjuvant before the first injection of laying hens. At weeks 2, 4 and 6, chickens received booster immunisations with the same

amount of protein emulsified in Freund's incomplete adjuvant. A control non-immune sample was obtained from the eggs laid by the same chickens prior to immunisation. Eggs were collected daily for the duration of the experiment and stored at 4°C until examined for the presence of IgY antibodies.

Chicken antibodies are passed on to the egg yolk to confer passive immunity to the developing chick. Consequently, egg yolks of immunized chickens provide a convenient source of antibodies. Immunoglobulins (IgY) are then easily isolated from the chicken egg yolk by PEG precipitation (Polson *et al.*, 1985). This circumvents the trauma that laboratory animals such as rabbits are subjected to during periodic bleeding to obtain anti-sera. Whereas chicken antibody (IgY) is obtained from egg yolk, rabbit IgG antibody is isolated from mammalian serum. Both IgY and IgG were purified to apparent homogeneity using the polyethylene glycol (PEG; Mr 6000) precipitation method. PEG (M_r 6000) is a mild precipitating agent that operates on a steric exclusion mechanism whereby proteins are concentrated in the extrapolymer space, until they exceed their solubility limits. The methods used in isolating IgG and IgY are essentially as described by Polson *et al.* (1964) and Polson *et al.* (1985) respectively.

Antibody against chicken IgY was raised in rabbits. Purified non-specific chicken IgY (100 μg) was emulsified in complete adjuvant and injected intradermally into a rabbit. Then two doses of 50 μg antigen in incomplete Freund's adjuvant were given 3 weeks and 5 weeks after the initial injection. Blood samples were obtained from the animal through the marginal ear vein 10 days after the third injection. The blood samples were allowed to carefully drip by the sides of the tube to enable the serum to separate out.

2.10.1 Reagents

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.2H_2O$ (15.6 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist. H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre.

2.10.2 Procedure for isolation of IgY from chicken egg yolks

Chicken egg-yolks were separated from the egg white and carefully washed under running water to remove traces of egg-white proteins. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer, pH 7.6, was added and mixed thoroughly. Solid PEG (M_r 6 0000, 3.5% (m/v) was dissolved in this mixture by gentle stirring. The resulting precipitated vitellin fraction was removed by centrifugation (4 420 x g, 30 min, 25°C), and the supernatant fluid filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% [i.e. 8.5% PEG (m/v) added]. After the PEG had dissolved, the sample was centrifuged (12 000 x g, 10 min, room temp). The supernatant was removed and the pellet re-dissolved in 100 mM Na-phosphate buffer, pH 7.6, in a volume equal to the volume obtained after the initial filtration. PEG was again added to make the final concentration of 12% (m/v) PEG. The PEG was dissolved and the precipitated protein collected by centrifugation (12 000 x g, 10 min, 25°C). The supernatant fluid was discarded and the final antibody pellet dissolved in 1/6 of the original egg yolk volume, using 100 mM Na-phosphate buffer, pH 7.6, and stored at 4°C.

2.10.3. Procedure for isolation of IgG from rabbit serum

One volume of rabbit serum was mixed with two volumes of borate buffered saline. Solid PEG (M_r 6 000) was added to the diluted serum to 14% (m/v), dissolved with constant stirring and the mixture was centrifuged (12 000 x g, 10 min, 25°C). The pellet was re-dissolved in the original serum volume, using 100 mM Na-phosphate buffer, pH 7.6. Again PEG was added to make a 14% (m/v) PEG solution. After the PEG was dissolved, the solution was re-centrifuged (12 000 x g, 10 min, room temp). Finally, the pellet was re-dissolved in 100 mM Na-phosphate buffer, pH 7.6, containing 60% (v/v) glycerol. The final volume was adjusted to be equal to half the original serum volume. The samples were stored at -20^{0}C.

2.10.4 Determination of IgY and IgG concentrations

The A_{280} of IgY and IgG solutions in 100 mM Na-phosphate buffer were determined and the concentrations of IgY and IgG calculated using the equation $A_{280} = Ecl$, where;

A_{280} is the absorbance at 280 nm,
c is the concentration of the absorbing species
l is the light path length and,

E is the molar extinction coefficient

[extinction coefficient of IgY, E_{280} (1 mg/ml) = 1.25 (Coetzer,1985)] and that of IgG, E_{280} (1 mg/ml) = 1.43 (Hudson and Hay, 1980). It was usually necessary to dilute the immunoglobulin samples 1:40 before reading the A_{280} values.

2.11 Western blotting

This technique was used to identify and characterise proteins separated by electrophoresis. The technique was also used to evaluate the specificity of antibodies raised against the purified proteins. The procedure was adapted from Towbin *et al.* (1979). Methanol was included in the transfer buffer to increase binding of the protein-SDS complexes to the nitrocellulose membrane. Anionic detergents such as SDS have a tendency to denature antigenically reactive sites (epitopes) on fractionated molecules. This limitation may be severe when screening panels of monoclonal antibodies since such antibodies are directed against single epitopes within an antigenic molecule. However, denaturation was not considered to be a major problem in the present investigation because polyvalent monospecific antibodies were utilised. The detection system consisted of an enzyme labelled (e.g. horse radish peroxidase) secondary antibody directed against a primary antibody. The enzyme catalyses a reaction leading to the formation of a precipitating coloured product on the nitrocellulose membrane.

2.11.1 Reagents

Blotting buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Tris (9.08 g) and glycine (43.2 g) were dissolved in 2 litres of dist. H_2O, and methanol (600 ml) was added. The volume was made up to 3 litres with dist.H_2O. SDS [3 ml of 10% (m/v) solution, Section 2.6.1] was added prior to use.

Tris buffered saline (TBS) [20 mM Tris, 200 mM NaCl, pH 7.4]. Tris (2.42) and NaCl (11.69) were dissolved in 950 ml of dist. H_2O the pH adjusted to 7.4 with HCl, and made up to 1 litre.

Antibody diluent (0.5% BSA-TBS). BSA (0.5 g) was dissolved in (TBS 100 ml).

Blocking solution (5% non-fat milk powder). Non-fat milk powder (5 g) was dissolved in TBS.

Ponceau S stain for protein bands on nitrocellulose. Ponceau S [0.1% (m/v)] was prepared in 1% (v/v) glacial acetic acid.

4-chloro-1-naphthol substrate solution [0.06% (m/v) 4-chloro-1-naphthol, 0.01% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.03 g) was dissolved in methanol (10 ml). Two ml of this solution was diluted to 10 ml with TBS, with addition of 30% hydrogen peroxide (4 µl).

BCIP/NBT substrate solution. One 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablet was dissolved in 10 ml of dist. H_2O.

Enzyme-linked secondary antibodies. A commercial rabbit anti-chicken IgG linked to alkaline phosphatase purchased from Sigma, was used for western blots. This antibody cross-reacted with chicken IgY on blots. Initially, before commercial enzyme-labelled anti-chicken antibodies were readily available, rabbit anti-chicken IgY was conjugated to horse radish peroxidase (HRPO). The conjugation of HRPO to immunoglobulins was carried out according to Hudson and Hay (1980). HRPO (4 mg) was dissolved in 1 ml of dist.H_2O and 200 µl of freshly prepared 100 mM sodium periodate solution was added. The mixture was stirred for 20 min at room temperature. It turned a greenish-brown colour. The mixture was dialysed against 1 mM Na-acetate buffer, pH 4.4, overnight at 4°C. The pH was raised to approximately 9–9.5 by addition of 200 mM Na_2CO_3 buffer, pH 9.5 (20 µl), and 1 ml of an 8 mg/ml IgG fraction immediately added. This solution was left at room temperature for 2 h. Freshly prepared 4 mg/ml Na-borohydride solution (100 µl) was added and the solution left at 4°C for 2 h to reduce any free enzyme. The mixture was dialysed against Na-borate buffer, overnight at 4°C, before an equal volume of 60% glycerol in Na-borate buffer was added and the conjugate stored at 4°C. The dilution of conjugate to be used was established in a checkerboard ELISA. A suitable working dilution (1 in 350) of primary antibody was obtained from a steep part of the antibody titration curve, sufficiently high above background values.

2.11.2 Procedure

Both native-and SDS-PAGE were usually performed on duplicate gels. One gel was stained to show the total protein pattern while the other was used for blotting. The electrophoretic protein transfer procedure (electroblotting) was adapted from Burnette

quantitative recovery of proteins from SDS-containing polyacrylamide gels. Nitrocellulose paper, Hybond C (Amersham), was used. Electrophoretic transfer was carried out for 16 h at 30 V. The apparatus was connected to a refrigerated water circulator kept at 8°C. The buffer was stirred constantly by a magnetic stirrer to ensure even distribution of the cooling buffer. After 16 h, the sandwich was removed and the filter paper peeled off the gel. The gel was carefully removed and stained with Coomassie Blue R 250 (Section 2.6) to assess the efficiency of electroblotting.

The nitrocellulose sheet removed from the filter paper was air dried for about 1.5 h. The nitrocellulose sheet was transiently stained in Ponceau S for 30-60 sec, rinsed in dist.H_2O, the positions of the MW markers marked with a pencil, and the sheet cut into strips as required before destaining the strips by addition of a drop of 500 mM NaOH to the dist.H_2O. Unbound sites on the nitrocellulose strips were blocked for 1 h with 5% (m/v) low fat milk powder in TBS at 25°C, washed in TBS (3 x 5 min) and incubated for 2 h with primary antibody in 0.5% BSA-TBS. The strips were washed in TBS (3 x 5 min), incubated in HRPO-linked secondary antibody, diluted in antibody diluent, for 1 h at 25°C, and again washed in TBS (3 x 5 min). The strips were immersed in substrate solution and allowed to react in the dark until bands were clearly evident against a lightly stained background. Finally, the strips were removed from the substrate solution, washed in dist.H_2O and dried between filter paper. This last step ensured good preservation of the bands before photography.

2.12 Enzyme-linked Immunosorbent Assay

A modified enzyme-linked immunosorbent assay (Coetzer *et al.*, 1991) was used to test the reactivity of the antibody raised against the purified protein. Routine screening assays were done to evaluate the progress of polyclonal antibody production during the immunization period. This technique measures quantitative properties of antibodies and so complements Western blotting, which gives qualitative information about antibody specificity.

2.12.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2) were dissolved and made up to 1 litre with dist. H_2O.

Antibody diluent. See Section 2.11.1

0.5% (m/v) Bovine serum albumin-PBS (BSA-PBS). BSA (0.5 g) was dissolved in 100 ml of PBS.

0.1% (v/v) PBS-Tween. Tween 20 (1 ml) was made up to 1 litre in PBS.

0.15M Citrate-phosphate buffer, pH 5.0. A 0.15 M solution of citric acid (22.9 g/l) was titrated with a 0.15 M solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (m/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate phosphate buffer, pH 5.0]. ABTS (7.5 mg) and H_2O_2 (7.2 µl) were dissolved in citrate phosphate buffer, pH 5.0 (15 ml) for one ELISA plate.

Stopping reagent [Citrate-phosphate-0.1% (m/v) NaN_3]. NaN_3 (0.1 g) was dissolved and made up to 100 ml in citrate-phosphate buffer.

2.12.2 Procedure

The purified enzyme or VSG (0.1 µg in 100 µl of PBS) was incubated in 96-well Nunc-Immuno MaxiSorp™ microtitre plates overnight at 4°C. The plates were washed in PBS-Tween [0.1% (v/v) Tween 20 in PBS]. After washing, 200 µl of 0.5% BSA-PBS was added to each well and incubated for 1 h at 37°C to prevent the non-specific binding of antibody to the unoccupied spaces in wells in subsequent reactions. The plates were washed in PBS-Tween and drained. Serial two-fold dilutions of primary antibody (starting from 1 mg/ml) were prepared in the plate in 0.5% BSA-PBS and incubated (100 µl, 2 h at 37°C). The plates were then washed three times with PBS-Tween, and incubated for 1 h at 37°C with 100 µl of rabbit anti-chicken IgY conjugated to horseradish peroxidase diluted in 0.5% BSA-PBS. After washing, the peroxidase activity was measured with 100 µl of substrate solution in citrate-phosphate buffer, added to each well. The colour was allowed to develop (10-15 min) in the dark. The enzyme reaction was stopped by addition of 50 µl of stopping reagent. Absorbance of each well was read at 405 nm on a Titertek ELISA plate reader and compared against the background of control samples.

Chapter 3

Purification and characterisation of 20S proteasome from *Trypanosoma brucei*

3.1 Introduction

The success of trypanosomes in their mammalian hosts is attributed to their remarkable ability to evade their host's specific immune response by altering their VSG coat. Lateral movement of VSG/anti-VSG complexes was detected in earlier studies on the surfaces of trypanosomes (Russo *et al.*, 1993). This movement was directed from the surfaces of the parasites into their lysosomes via their flagellar pockets. By using mixtures of protease inhibitors, which blocked protein breakdown, proteases were implicated in the internalisation and processing of the VSG/antibody complexes. Endocytosis is a crucial event in the physiology of trypanosomes and recent experimental data have identified the ubiquitin-proteasome system as a regulator of endocytosis (Strous *et al.*, 1996; Strous and Gover, 1999). Even more interesting is the involvement of a lactacystin-inhibitable protease complex in the quality control of glycophosphosphatidylinositol (GPI) anchor addition to proteins (Oda *et al.*, 1996; Wilbourne *et al.*, 1998). In trypanosomes, GPI anchors VSG on the surface of the plasma membrane of the cell (Englund, 1993). Previous studies by Russo *et al.* (1993) showed that chymostatin, an inhibitor of chymotrypsin-like proteases, blocked endocytosis of VSG/anti-VSG complexes. Hence, we decided to determine whether *T. b. brucei* possessed chymotrypsin-like activity and to purify and characterise this activity.

Most studies on enzymes require pure preparations of the enzyme. Pure enzyme may be obtained by isolating the enzyme either from cell homogenates or from expression systems following cloning of the gene segment coding for the protein. The latter approach is based upon the use of conserved gene sequences and requires detailed knowledge of the primary sequence of the protein. Since this information was not available, chymotrypsin-like activity was isolated directly from *T. brucei* lysates. A new isolation method was developed to achieve this.

Interestingly, characterisation of the partially purified protease revealed properties suggesting that it was a high molecular mass, multicatalytic proteinase [also called the proteasome (Coux *et al.*, 1996, Tanaka *et al.*, 1998)]. For convenience, the multicatalytic proteinase isolated from *T. brucei* and from bovine red blood cells will be referred to as MCP-Tb and MCP-rbc respectively. MCP-rbc was isolated for comparative studies because it is the host equivalent of the parasite MCP-Tb. While this study was underway, proteasomes were isolated from *T. brucei* by another laboratory (Hua *et al.*, 1996, Mutomba *et al.*, 1997, To and Wang, 1997), but the two studies differ in many aspects. These differences and their importance will be addressed in the relevant sections.

3.2 Reagents

Fresh bovine blood. Citrated fresh bovine blood was obtained from Cato Ridge Abattoir.

Lysis buffer (10 mM Tris-HCl, 100 μM Na_2EDTA, 1 mM DTT, 50 μM E-64, 2 mM ATP, pH 7.0). Tris (0.1211 g), Na_2EDTA (0.0037 g), DTT (0.0154 g), E-64 (0.2 mg) ATP (0.1102 g) were dissolved in about 80 ml of dist. H_2O titrated to pH 7.0 with HCl and made up to 100 ml with dist .H_2O.

Standard buffer G [10 mM Tris-HCl, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 100 μM Na_2EDTA, 1 mM DTT, 20% glycerol (v/v), pH 7.0)]. Tris (1.211 g), KCl (1.864 g), NaCl (0.584 g), $MgCl_2$ (0.223 g), Na_2EDTA (0.037 g), DTT (0.154 g), glycerol (200 ml) were dissolved in about 950 ml of dist. H_2O titrated to pH 7.0 with HCl and made up to 1000 ml with dist. H_2O.

Standard buffer H (20 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.6). Tris (9.68 g), NaCl (6.58 g), Na_2EDTA (1.489g), β-mercaptoethanol (280 μl), were dissolved in about 3500 ml of dist. H_2O titrated to pH 7.6 with HCl and made up to 4 litres.

Equilibrating buffer A (Standard buffer G containing 100 mM NaCl). NaCl (5.84 g) was added to 950 ml of standard buffer, titrated to pH 7.0 with HCl and made up to 1000 ml with dist.H_2O.

Elution buffer A (Standard buffer G containing 500 mM NaCl). NaCl (29.22 g) was added to 950 ml of standard buffer, titrated to pH 7.0 with HCl and made up to 1000 ml with dist.H_2O.

Equilibrating buffer B [10 mM potassium phosphate, 1 mM DTT, 20% glycerol (v/v), pH 6.8]

KH_2PO_4 (1.361 g), DTT (0.154 g) and glycerol (200 ml) were dissolved in about 950 ml of dist. H_2O, titrated to pH 6.8 with KOH and made up to 1000 ml with dist. H_2O.

Elution buffer B [300 mM potassium phosphate, 1 mM DTT, 20% glycerol (v/v), pH 6.8]. KH_2PO_4 (8.1654 g), DTT (0.0308 g) and glycerol (40 ml) were dissolved in dist. H_2O, titrated with KOH to pH 6.8 and made up to 200 ml with dist. H_2O.

3.3 Procedures

3.3.1 Purification of MCP-Tb

All enzyme purification procedures were carried out at 4°C. *T. b. brucei* were propagated and isolated as outlined in Section 2.3.2. Cells (approximately 10^{10} trypanosomes) were suspended in lysis buffer (20 ml) and ruptured in a French pressure cell (Aminco Silver Springs) at approximately 2 500 Psi. E-64 and EDTA were included in the lysis buffer to minimize possible proteolytic degradation of MCP-Tb during the purification process by cysteine and metalloproteases. The cell lysate was centrifuged (100 000 x g, 1 h, 4°C) in a fixed-angle 75 Ti rotor and the supernatant collected. The pellet was washed twice by re-suspension in lysis buffer (20 ml), followed by centrifugation (100 000 x g, 1 h, 4°C). The original supernatant and wash supernatants were combined. Glycerol was added to the pooled supernatant [20% (v/v) final conc.] and the mixture loaded onto a Q-Sepharose anion exchange column (2.5 x 10 cm), equilibrated with 5 bed volumes of standard buffer G (30 ml. h^{-1}). Unbound proteins were eluted with equilibrating buffer A. Bound proteins were eluted with a linear gradient of NaCl from 100-500 mM (400 ml) in buffer A. Fractions active against Z-Gly-Gly-Leu-AMC were pooled and concentrated (5-10 fold) by dialysing (using Mr 12 000 cut-off dialysis membrane) against dry polyethylene glycol (PEG), 20 000.

The concentrated, active fraction from the Q-Sepharose column (2 ml) was applied to a Sephacryl S-300 column (2.5 x 87 cm) equilibrated with buffer A. A higher concentration of NaCl (100 mM) was necessary to avoid non-specific interaction between the protein and the resin. In this step, low molecular mass contaminants were removed from the proteinase complex. The enzyme eluted within the fractionation range of the beads as a single peak of Z-Gly-Gly-Leu-AMC hydrolysing activity (Section 3.4.1). This allowed the relative molecular mass of the purified protein to be estimated by comparison with the elution positions of molecular mass markers. These were thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B-12 (1.35 kDa). The void volume of the column (V_0) was determined using Dextran Blue (>2 000 kDa).

The active fractions obtained from the Sephacryl S-300 column were pooled and dialysed (16 h, 4°C) against three changes of equilibrating buffer B (3 x 1 litre). The dialysed sample was loaded onto an HA-Ultrogel column (1.5 x 12 cm) equilibrated (20 $ml.h^{-1}$) with equilibrating buffer B. HA-Ultrogel is a microcrystalline hydroxyapatite (40% by mass) embedded in cross-linked 4% beaded agarose. This form of hydroxylapatite for adsorption chromatography avoids many of the problems posed by hydroxylapatite itself. It needed no pre-cycling for removal of fines and had satisfactory flow rates. The hydroxylapatite column was washed with equilibrating buffer B until the absorbance reached baseline before adsorbed material was eluted with 200 ml of a linear gradient, 10-300 mM phosphate, in elution buffer B. The fractions containing hydrolysing activity were pooled and the glycerol concentration diluted to 5% (v/v) (1-5 mg protein) by addition of assay buffer (Section 3.4.1.1). The sample was layered onto a linear 10-40% (v/v) glycerol gradient in standard buffer G. After centrifugation (100 000 x g, 22 h, 4°C) in a SW 40 rotor, the enzyme was concentrated by dialysis against dry polyethylene glycol 20 kDa using 12 kDa cut-off limit dialysis membrane. The glycerol concentration was brought to 20% (v/v) and the purified enzyme stored at -20°C. The activity of the enzyme was stable at this temperature for several months. However, when a previously stored sample was used in non-denaturing gel electrophoresis, a smear instead of a sharp protein band was obtained implying that the complex itself may not remain intact after long storage.

3.3.2 Purification of MCP-rbc

The multicatalytic proteinase from bovine red blood cells (bovine MCP-rbc) was purified using a procedure adapted from several sources (Dahlmann *et al.*, 1985; Kanayama *et al.*, 1992; Chu-Ping *et al.*, 1994). Bovine blood (1 000 ml) was collected in 2% (m/v) citrate and cells sedimented by centrifugation (2 000 x g for 1 h). The supernatant and the buffy coat were removed by aspiration. The red cells were resuspended in four volumes PBS and re-centrifuged (2 000 x g; 1 h). The red cells were lysed by stirring (10 min) three volumes of standard buffer H with one volume of the packed red blood cells. The total lysate was centrifuged (13 000 x g: 1 h) and the supernatant collected. The pellet was resuspended in three volumes of standard buffer H, recentrifuged and this supernatant added to the first. One portion, "Fraction I" of this crude lysate was dialysed against buffer G for subsequent isolation of MCP-rbc from the erythrocytes. The second portion, "Fraction II" (for isolation of PA700) was subjected to batch ion-exchange chromatography on DEAE cellulose (See Section 6.2.2). This enabled the simultaneous purification of MCP-rbc and PA700 from the same starting material for a parallel experiment (Section 6.2).

Fraction I, previously dialysed against standard buffer G and stored at –20°C, was thawed and fractionated by the addition of solid $(NH_4)_2SO_4$ to 45% (m/v) saturation. Precipitated proteins were collected by centrifugation (15 000 x g, 20 min, 25°C) and discarded. The supernatant was saturated to 65% (m/v) with respect to $(NH_4)_2SO_4$. The mixture [45-65% (m/v) $(NH_4)_2SO_4$ saturation] was centrifuged (15 000 x g, 20 min, 25°C). The supernatant was discarded and the pellet dissolved in about 40 ml of standard buffer G, and dialysed against three changes of buffer B (3 x 2 litres, 16 h, 4°C).

The dialysed enzyme solution was chromatographed on Q-Sepharose as described for MCP-Tb in Section 3.3.1. The fractions containing Z-Gly-Gly-Leu-AMC hydrolysing activity were pooled, dialysed against dry PEG, 20 kDa and further purified on Sephacryl S-300 and HA-Ultrogel as described for MCP-Tb in Section 3.3.1. The Z-Gly-Gly-Leu-AMC active pool from HA-Ultrogen was diluted to 5% (v/v) glycerol using assay buffer (Section 3.4.1.1) and concentrated using dry 20 kDa PEG to approx. 1 ml. Approximately 1-2 mg protein (500 µl) was layered onto a linear 10-40% (v/v) glycerol gradient in standard buffer G. The gradient was centrifuged (100 000 x g, 22 h, 4°C) in a SW 40 rotor. Fractions (1 ml) were collected by draining the tube from the top with the aid of a

peristaltic pump. The fractions were assayed for activity against Z-Gly-Gly-Leu-AMC and active fractions pooled. The active pool was concentrated by dialysis against dry 20 kDa PEG. The glycerol concentration was brought to 20% and the sample purity evaluated by native gradient (5-10%) PAGE (Section 2.6.2).

3.4 Enzyme Assay

3.4.1 Assay with 7-amido-4-methylcoumarin substrates

A variety of commercially available synthetic peptides were used to determine peptidase activities of MCP. Peptides with the 7-amido-4-methylcoumarin (AMC) leaving group provided the most sensitive substrates. These substrates have been used at concentrations below their K_m values of 0.1-1 mM reported for mammalian proteases (Rivett, 1989). Z-Gly-Gly-Leu-AMC was routinely used to measure the chymotrypsin-like activity, whereas either H-Pro-Phe-Arg-AMC or Z-Gly-Gly-Arg-AMC was used to assay for trypsin-like activity of the proteinase.

3.4.1.1 Reagents

Assay buffer (60 mM Tris-HCl, 20 mM KCl, 10 $MgCl_2$, 1 mM DTT, 1 mM ATP, pH 8.0). Tris (0.726 g), KCl (0.149 g), $MgCl_2$ (0.203 g), DTT (0.031 g) and ATP (0.055 g) were dissolved in 80 ml of dist. H_2O, titrated to pH 8.0 with HCl, and made up to 100 ml with dist. H_2O.

Substrate stock solutions. Stock solutions were made to a concentration of 2 mM in dimethyl sulfoxide (DMSO) and stored at -20°C. Stock solutions were usually diluted in assay buffer to 0.1 mM just before use.

3.4.1.2 Procedure

This assay system was essentially as described by Dahlmann *et al.* (1995). Briefly, the reaction mixture (in a total volume of 100 µl) consisted of assay buffer (50 µl), peptide-AMC (25 µl; 0.2 mM) and enzyme (0.1-0.2 µg protein). Reactions were initiated by addition of either substrate or enzyme and hydrolytic rates were measured at 37°C by continuously monitoring increases in fluorescence (excitation 360 nm; emission 460 nm) in a spectrofluorometer (7620 Microplate Fluorometer, Cambridge Technology, Inc.).

Control assays contained no enzyme. All assays were inspected for reaction linearity. Enzyme concentrations were adjusted to allow for measurement of initial velocities only. The changes in fluorescence intensity were converted to micromoles of AMC by determining, under the same conditions, the fluorescence of a serially diluted standard solution of AMC.

3.4.2 Assay with Z-Leu-Leu-Glu-β-naphthylamide substrate

The peptide Z-Leu-Leu-Glu-β-naphthylamide (βNA) is routinely used to assay peptidyl glutamylpeptide hydrolase (PGPH) activity (Orlowski, 1990; Djaballah and Rivett, 1992). Hydrolysis of this substrate releases βNA, which can be measured directly in a fluorometer, or colorimetrically by coupling to a diazonium salt. The product βNA is thought to be carcinogenic and is not available commercially. A small amount was prepared by the total enzymatic digestion of Z-Leu-Leu-Glu-βNA to construct a standard curve.

3.4.2.1 Reagents

Assay buffer (40 mM Hepes, pH 8.0). Hepes (0.953) was dissolved in 80 ml of dist. H_2O, titrated with NaOH to pH 8.0 and made up to 100 ml with dist. H_2O.

Substrate stock solution (10 mM Z-Leu-Leu-Glu-βNA). Z-Leu-Leu-Glu-βNA (0.006 g) was dissolved in DMSO (1 ml). This substrate was freshly prepared when required.

Borate buffer (100 mM Na-borate, pH 7.4). Boric acid (0.618 g) was dissolved in 80 ml of dist. H_2O, adjusted to pH 7.4 with NaOH and made up to 100 ml with dist. H_2O.

3.4.2.2 Procedure

Peptidyl glutamylpeptide hydrolase activity was measured essentially according to Mykles and Haire (1991). The reaction mixture (200 µl) contained 1 mM Z-Leu-Leu-Glu-βNA (40 µl), 40 mM Hepes, pH 8.0 (100 µl) and approx. 0.2 µg enzyme and was incubated for 1 h at 37°C. The reaction was stopped by addition of 0.3 ml 1% (v/v) SDS and 1 ml borate buffer (pH 7.4). The amount of substrate hydrolysed was measured in a Hitachi F-2000 Fluorescence Spectrophotometer (excitation 336; emission 410 nm). Control samples contained no enzyme.

To prepare a standard curve 0.6 mM βNA was produced by the complete digestion of Z-Leu-Leu-Glu-βNA (200 µl; 0.6 mM) by *Staphylococcus aureus* V8 proteinase (1 µg) in 50 mM ammonium bicarbonate buffer, pH 8.0 (Rivett *et al.*, 1994). A standard curve was prepared using 40 mM Hepes, pH 8.0, βNA, 1% SDS and borate buffer as described above.

3.4.3 Degradation of β-casein substrate

The degradation of protein substrates is commonly assayed by measuring radiolabelled, acid-soluble products in a liquid scintillation counter. Alternatively, the degradation products of unlabelled protein may be assessed by use of SDS-PAGE. Degradation of β-casein by MCP-Tb and MCP-rbc were assessed on SDS-PAGE.

3.4.3.1 Reagents

Assay buffer. As per Section 3.4.1.1.

β-casein stock solution. Dephosphorylated β-casein (10 mg) was dissolved in assay buffer (1 ml). The substrate solution was centrifuged (12 000 x g, 1 min, 25°C) to remove undissolved material. The pellet was discarded and the protein concentration in the supernatant was estimated by the Bradford (1976) dye binding method (Section 2.4). Aliquotes (100 µl) were stored at –20°C.

3.4.3.2 Procedure

Caseinolytic activity of MCP-Tb and bovine MCP-rbc was determined by a gel-electrophoretic method (Yu *et al.*, 1991). Purified enzyme (0.2 mg/ml, 10 µl) was added to β-casein substrate (51.3 µg, 90 µl) and assay buffer (75 µl), in 1.5-ml microfuge tubes and incubated at 37°C. Aliquots of reaction mixture (25 µl) were removed after 0, 45 and 90 min and an equal volume of reducing treatment buffer (Section 2.6.1) added. After boiling for 5 min to inactivate the enzyme, the samples were analysed by SDS-PAGE (Section 2.6.2). Bands corresponding to the protein substrate and its degradation products were visualised by silver staining (Blum *et al.*, 1987, Section 2.6.3).

3.4.4 Peptidase Inhibition

Chymostatin, leupeptin, TLCK, and TPCK were each dissolved separately in DMSO at a stock concentration of 10 mM, and diluted out in 60 mM Tris-HCl pH 8.0. Z-Leu-Leu-

Leu-H precipitated out of solution soon upon addition of an aqueous buffer and the enzyme was thus incubated with this inhibitor before addition of assay buffer. α-chymotrypsin-trypsin inhibitor was dissolved in assay buffer (Section 3.4.1.1) to a concentration of 20 mg/ml. Benzamidine and 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) were dissolved in assay buffer at a stock concentration of 100 mM and 20 mM, respectively. Purified proteasome (0.2 μg; 5 μl) was incubated separately with each of the inhibitors at the specified final concentration for 30 min at 25°C, and assayed for peptidase activity (Section 3.4.1). Controls, performed in parallel, contained no inhibitors and, where appropriate, these incorporated the organic solvents used to prepare stock solutions of inhibitors.

3.4.5. Determination of pH stability

Enzyme stability is pH-dependent and the optimum stability of an enzyme may differ from the pH at which it is optimally active. Three main approaches are often used to study pH stability of an enzyme. The enzyme may be pre-incubated at various pH values and assayed at those values. This method assesses a combination of stability and activity at a particular pH. Alternatively, after pre-incubation at various pH values, the enzyme is assayed at its optimal pH. Lastly, hydrolysis of the substrate can be monitored continuously at various pH values with excess substrate and the decrease in velocity over time used to calculate the half-life of the enzyme at each pH. In this study the second method was used to assess influence of pH on stability of the proteasome. This method was chosen because it gives a better reflection of the effect of pH on stability without interference of pH on activity. The assay buffer was substituted with constant-ionic-strength Acetate/Mes/Tris buffer (Ellis and Morrison, 1982). Constant-ionic strength AMT buffer eliminates any effects attributable to buffer salts or differing ionic strength.

3.4.5.1 Reagents

AMT buffers [100 mM acetate, 100 mM MES, 20 mM Tris containing 4 mM Na_2EDTA]. Glacial acetic acid (1.72 ml), MES (5.86 g), Tris (7.27 g) and $Na_2EDTA.2H_2O$ (0.45 g) were dissolved in dist. H_2O (240 ml). This solution was divided into 12 aliquots (20 ml each) each of which was titrated with either HCl or NaOH to the appropriate pH. The volume of each aliquot was made up with dist. H_2O to 25 ml.

3.4.5.2 Procedure

The pH profile of each activity of the proteasome was determined in triplicate over a pH range of 3.0 to 12.0 using constant-ionic strength AMT buffer. Enzyme (5 µl) and AMT buffer (50 µl) at the required pH were incubated for 30 min at 25°C. The reaction was started by addition of 25 µl of 0.2 mM peptide-AMC substrate and the activity followed as described in Section 3.4.1. PGPH activity was measured using Z-Leu-Leu-Glu-βNA (0.2 mM) as described in Section 3.4.2. The addition of enzyme and substrate solutions did not significantly change the pH of the AMT buffer in the final reaction mixture.

The pH stability of the enzyme was determined by incubating the enzyme in AMT buffer of pH 3.0 to 12.0 for 1 h at 37°C. This was followed by addition of pH-8.0 assay buffer (Section 3.4.1) and Z-Gly-Gly-Leu-AMC or Z-Gly-Gly-Arg-AMC.

3.5 Results

3.5.1 Multicatalytic proteinase purification

The steps used for isolating multicatalytic proteinase (MCP) from *Trypanosoma brucei* are summarised in Table 3.1. The MCP-Tb was purified over 61-fold with an overall recovery of 7%. The first extract (high-speed centrifugation supernatant) contained approximately 40% of the total activity in the lysate but the specific activity decreased by over 40% compared to the starting material. The decrease in percentage recovery and specific activity possibly resulted from activity remaining in the pellet during centrifugation but could also be attributed to the removal of contaminating enzyme activity such as trypanopain and oligopeptidase that hydrolyse the peptide-AMC substrates (Mbawa *et al.*, 1992; Morty *et al.*, 1998). For this reason, the inclusion of the crude trypanosomal lysate in the purification table makes the enrichment factors and the yield appear small. Thus, a purification table beginning with high-speed soluble (HSS) material might be more accurate and would give an enrichment of 109 and an overall recovery of 17%. However, an important point would have been concealed that nearly 50% of the total Z-Gly-Gly-Leu-AMC activity remains in the pellet. Attempts to extract this activity from the pellet by solubilisation with detergents were unsuccessful since enzyme activity was lost. Anion exchange chromatography of the soluble supernatant fraction from high speed centrifugation on Q-Sepharose at pH 7.0 (Fig. 3.1) was a critical step in this purification procedure for other contaminating Z-Gly-Gly-Leu-AMC hydrolysing activities were eliminated in this step.

Although the cysteine protease inhibitor E-64 (Barret *et al.*, 1986), (50 μM) and the metalloprotease inhibitor EDTA (100 μM) were routinely included in the lysis buffer, it was necessary to fractionate the proteins on Q-Sepharose within two hours after cell lysis to minimise possible degradation of MCP-Tb by endogenous proteases. The lysis buffer also contained 2 mM ATP instead of 20% glycerol to maintain MCP-Tb integrity (Kanayama *et al.*, 1992) and allow for faster sedimentation of contaminating proteins in the trypanosome lysate during high speed centrifugation.

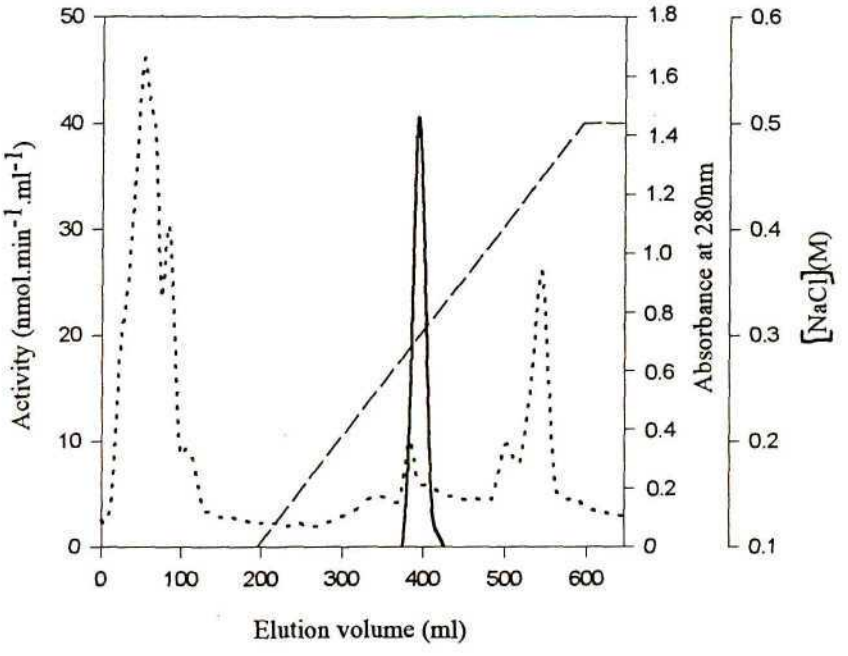

Figure 3.1. Anion exchange chromatography of trypanosomal MCP-Tb on Q-Sepharose.
Q-Sepharose (2.5 x 10 cm, 30 ml. h^{-1}) was equilibrated in standard buffer G [10 mM Tris-HCl, pH 7.0 containing 0.1 mM Na_2EDTA, 1 mM DTT, 25 mM KCl, 1.1 mM $MgCl_2$ and 20% glycerol]. After elution of unbound protein with standard buffer G containing 100 mM NaCl, bound protein was eluted with a linear gradient (----) of 100 to 500 mM NaCl in standard buffer G over 5 column volumes and fractions (9 ml) collected. (.....) A_{280}; (—) enzyme activity against Z-Gly-Gly-Leu-AMC.

The pI vaues of the subunits of MCP isolated from rabbit reticulocytes range between 5.2 and 5.4 (Hough *et al.*, 1987). The charge on the MCP-Tb complex is the summation of individual net charges of the subunits. Assuming a high degree of similarity in the amino acid composition between rabbit reticulocyte MCP and MCP-Tb, MCP-Tb should be negatively charged at pH 7.0. The MCP-Tb complex apparently has a net negative charge at pH 7.0. This would explain why it binds to Q-Sepharose. Contaminating proteinases

such as trypanopain probably elutes in the unbound fraction because trypanopain-Tc has a pI of 7.4 (Mbawa *et al.*, 1992) and should be weakly positively charged or neutral at pH 7.0. The bound fraction was eluted from the column at about 350-380 mM NaCl. The elution pattern of the proteins and the proteinase was essentially similar to that found in MCP-rbc purification. The total recovery of MCP-Tb was about 20%. It should be noted that the fractions showing peak activity against Z-Gly-Gly-Leu-AMC degraded several other peptide substrates including Z-Phe-Arg-AMC, Z-Gly-Gly-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-βNA. These are the activities associated with cysteine, trypsin-like and chymotrypsin-like proteinase, and peptidyl glutamylpeptide hydrolase (PGPH) activities (Mbawa *et al.*, 1992; Rivett, 1993; Orlowski *et al.*, 1993). The activity eluted from the Q-Sepharose column was subjected to molecular exclusion chromatography on Sephacryl S-300 (Fig. 3.2).

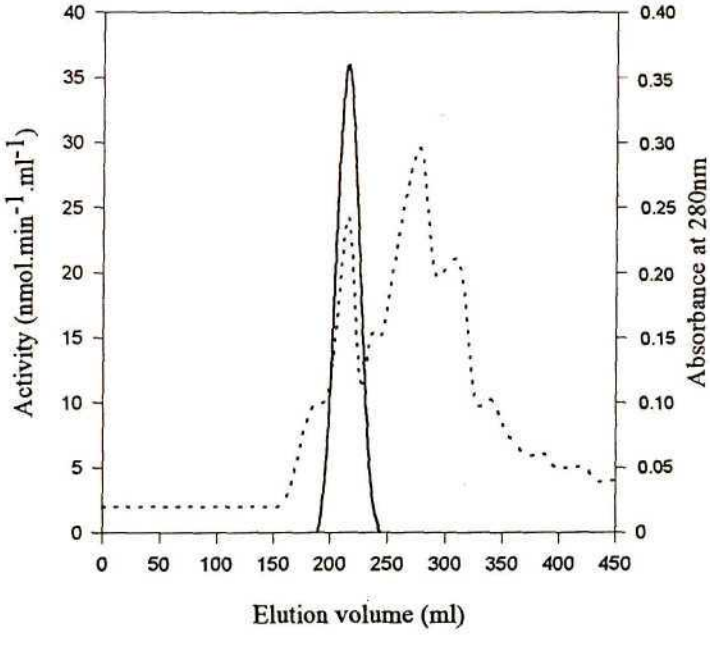

Figure 3.2. Molecular exclusion chromatography of trypanosomal MCP-Tb on Sephacryl S-300
Column, 2.5 x 87 cm (427 ml bed volume); standard buffer G [10 mM Tris-HCl, pH 7.0 containing 0.1 mM Na_2EDTA, 1 mM DTT, 25 mM KCl, 1.1 mM $MgCl_2$and 20% glycerol], flow rate 36 ml. h^{-1} (7.3 cm. h^{-1}); and fractions, 9 ml. Void volume was 167 ml. (....) A_{280}; (—) enzyme activity against Z-Gly-Gly-Leu-AMC. The column was calibrated under similar conditions with protein standards; thyroglobulin, M_r 670 kDa, bovine gamma globulin, M_r 158 kDa, chicken ovalbumin, M_r 44 kDa, equine myoglobin, M_r 17 kDa and vitamin B_{12}, M_r 1.35 kDa.

The gel-filtration column effectively eliminated low molecular mass contaminants. This step also served as a buffer change step for the subsequent purification step. The enzyme eluted close to the void volume as a single symmetrical peak of activity. The elution volume (V_e) of the enzyme was 189 ml whereas V_0 was 167 ml. Molecular mass analysis using molecular mass standards on the same column indicated a high relative molecular mass of 670 kDa for the Z-Gly-Gly-Leu-AMC activity. Pooled fractions from the Sephacryl S-300 column were applied directly to a hydroxylapatite column (Fig. 3.3).

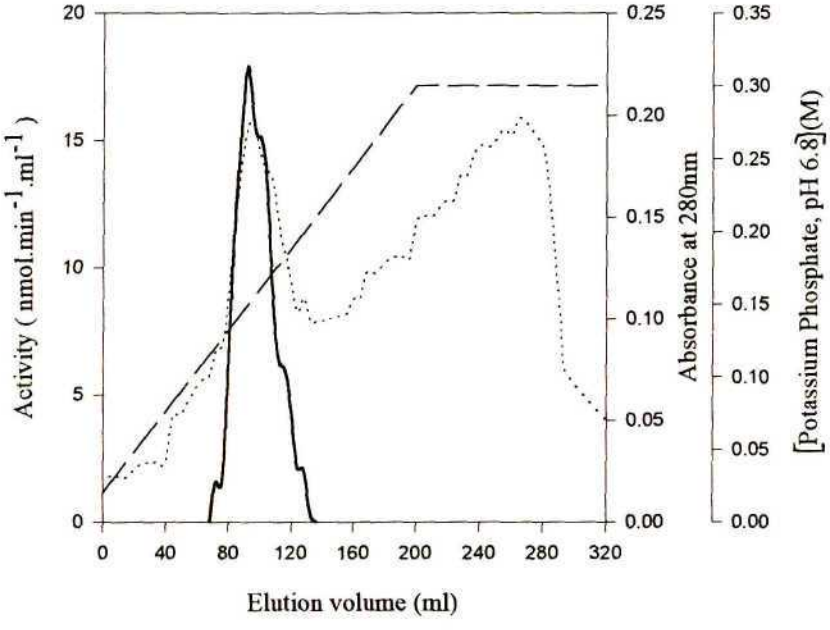

Figure 3.3. Hydroxyapatite column chromatography of trypanosomal MCP-Tb

HA-Ultrogel (1.5 x 12 cm, 20 ml. h^{-1}) was equilibrated in equilibration buffer B [10 mM potassium phosphate, 1 mM DTT, 20% (v/v) glycerol]. After elution of unbound proteins with equilibration buffer B, bound protein was eluted with a linear gradient (----) of 10-300 mM in buffer B over 10 bed volumes and fractions (4 ml) collected. (.....) A_{280}, (—) enzyme activity against Z-Gly-Gly-Leu-AMC.

Potassium phosphate was chosen as elution buffer because of the poor solubility of sodium phosphate salts at low temperatures. The active fractions were eluted from HA-Ultrogel at 150 mM potassium phosphate. A sharp peak of activity coincided with a small protein peak. The total recovery from the HA-Ultrogel column was 10% and resulted in a 31-fold purification.

The final purification was achieved by glycerol density gradient (10-40%) sedimentation. The specific activity of the purified MCP-Tb was 7.5 $nmol.min^{-1}.mg^{-1}$ protein against Z-

Gly-Gly-Leu-AMC substrate of MCP-Tb, with a 61-fold purification and 7% recovery of activity. Both the yield and specific activity were greater than those of bovine MCP-rbc (Table 3.1). Bovine MCP-rbc was purified 171-fold in a five-step procedure with a yield of 3%. The recovery of Z-Gly-Gly-Leu-AMC hydrolysing activity was low after the $(NH_4)_2SO_4$ fractionation step. This low yield, however, appears not to be due to loss in the MCP-rbc activity but is probably due to the presence in the crude extract, of other endogenous proteinases with activity towards the Z-Gly-Gly-Leu-AMC substrate.

Table 3.1. Summary of the purification of the MCP-Tb from *T. brucei* and MCP-rbc from bovine red blood cells.
All activities were determined using Z-Gly-Gly-Leu-AMC at a final concentration of 50 μM.

Purification step	Total protein (*mg*)	Total activity (*pmol.min*$^{-1}$)	Specific activity (*pmol.min.*$^{-1}$*.mg*$^{-1}$)	Purification (*-fold*)	Yield (%)
MCP-Tb(10^{10} trypanosomes)					
Lysate	279.00	34200	122.6	1	100
HSS (100 000 x g, 1h)	203.00	14000	69.0	(0.6)	41
Q-Sepharose	8.07	6970	864.0	7	20
Sephacryl S-300	1.92	6130	3193.0	26	18
HA-Ultrogel	0.87	3304	3780.0	31	10
Dens. grad. sedimentation	0.31	2313	7510.0	61	7
MCP-rbc (1000 ml blood)					
Crude extract (Fraction I)	13715.00	246560	18.0	1	100
$(NH_4)_2SO_4$ (45-65%)	3508.00	28614	8.2	(0.5)	12
Q-Sepharose	72.15	19540	270.8	15	8
Sephacryl S-300	23.23	12937	556.9	31	5
HA-Ultrogel	5.48	9788	1786.1	99	4
Dens. grad. sedimentation	2.36	7253	3073.3	171	3

HSS = High speed supernatant

The purification factor (171-fold) and yield (3%) of MCP-rbc obtained in this study are consistent with the purification factor (173-fold) and yield (5.5%) of rat skeletal muscle-MCP purified using Z-Val-Gly-Arg-AMC to monitor purification (Dahlmann *et al.*, 1985). However, the results differ from the enrichment (34.3-fold) and protein recovery (1.0%) of rat skeletal muscle-MCP using Suc-Ala-Ala-Phe-AMC to monitor activity (Dahlmann *et al.*, 1985). MCP-rbc showed a higher purification factor than human MCP from liver (115-

fold) using Z-Gly-Gly-Arg-AMC (Mason, 1990), but comparatively lower than the purification factor (380.4) and yield (7.0%) obtained using Suc-Leu-Leu-Val-Tyr-AMC to monitor MCP purification from rabbit reticulocytes (Hough *et al.*, 1987). The variation in activity and protein recovery may result from different purification protocols or difference in cell types constituting the starting material. MCP has been estimated to constitute up to 1% of the mammalian cell protein content (Tanaka *et al.*, 1986), but concentration varies considerably among cell types. The MCP concentration is greater in organs [e.g. bovine pituitary (Pereira *et al.*, 1992; Eleuteri *et al.*, 1997)] where average rates of protein breakdown are greater than in other tissues [e.g. skeletal muscle (Dahlmann *et al.*, 1995)]. A comparison of the specific activities obtained suggests that MCP is more abundant in *T. brucei* (at least 2.4-fold) than it is in bovine erythrocytes and this is consistent with the high levels of activity and rapid cell division rates of these organisms.

3.5.1.1 Subunit analysis

Enzyme homogeneity was assessed by native PAGE as outlined in Section 2.6.2. The enzyme migrated as a single band of about 590 kDa on a non-denaturing (5–10%) gradient gel (Fig. 3.4).

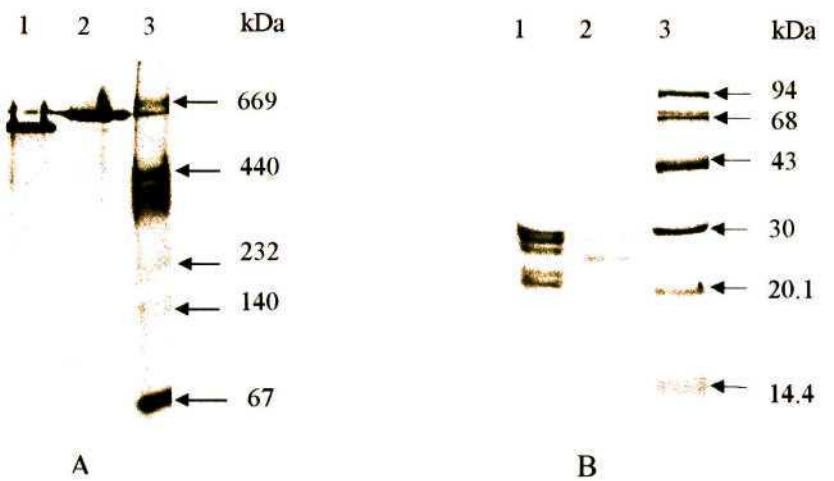

Figure 3.4. Evaluation of MCP-Tb and MCP-rbc purification by gradient PAGE and SDS-PAGE.
Panel A, non-denaturing (5-10% gradient) PAGE of MCP-Tb (lane 1), MCP-rbc (lane 2) and M_r markers (lane 3), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), LDH (140 kDa) and BSA (67 kDa). *Panel B*, reducing SDS-PAGE (12.5%) analysis of MCP-rbc (lane 1), MCP-Tb (lane 2) and M_r markers (lane 3), phosphorylase b (94 kDa), bovine serum albumin 68 kDa, ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) performed as described in Section 2.6.2 and the gels were silver stained.

The molecular mass obtained from gel electrophoresis was less than that for similar enzyme complexes reported in archaebacterium and yeast cells (Hilt and Wolf, 1992; Seemuller *et al.*, 1995). When analysed on reducing and non-reducing SDS-PAGE, the enzyme showed a polypeptide band pattern at 27, 26, 22 and 21 kDa. The protein bands obtained under reducing and non-reducing conditions appeared similar indicating that the subunits of the trypanosomal MCP-Tb are held together by hydrophobic interactions and do not appear to involve disulfide bonds.

3.5.1.2 Zymogram analysis

Gelatin zymogram analysis of purified MCP-Tb and MCP-rbc show that proteolytic activities are present only in intact native forms of the enzymes. The sites of proteinase activity are visible as clear bands against a stained gelatin background on the upper part of the gel (Fig.3.5). The MCP-Tb and MCP-rbc dissociate upon electrophoresis on SDS-PAGE to give protein subunits in the 18-30 kDa range. The proteinase activity is lost upon dissociation of the subunits.

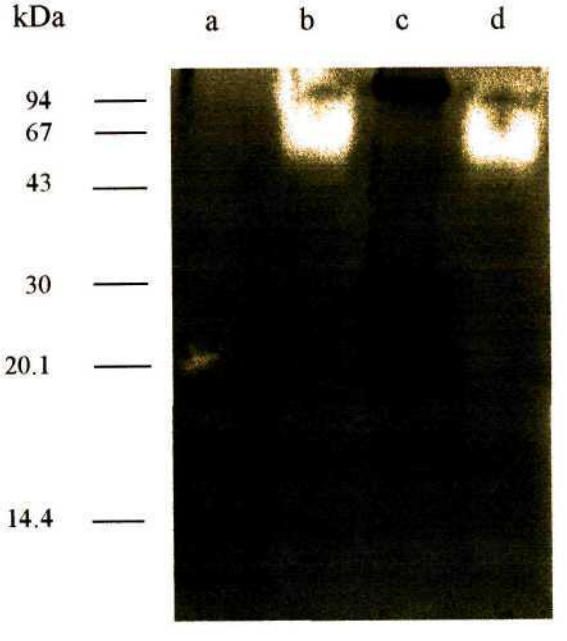

Figure 3.5. Gelatin-substrate SDS-PAGE analysis of MCP-Tb and MCP-rbc.

Purified MCP-Tb and MCP-rbc were electrophoresed on 10% SDS-PAGE co-polymerised with 0.1% (m/v) gelatin. *Lane a*, MCP-Tb plus inhibitor (Z-Leu-Leu-Leu-H), *lane b*, MCP-Tb alone, *lane c*, MCP-rbc plus inhibitor and *lane d*, MCP-rbc alone. The gel was incubated with assay buffer (Section 3.4.1) and stained with amido black as outlined in Section 2.7.2

Bands of activity were not seen in the presence of the proteasome specific inhibitor Z-Leu-Leu-Leu-H (5 μM) (Jensen *et al.*, 1995). Since there were no sites of proteolytic activity in lanes where the inhibitor was included, the proteinase activity of MCP-Tb, therefore, may not be independent from the peptide-hydrolysing sites of MCP-Tb. It is important to note that MCP-Tb clearly behaves like a proteinase, not merely a peptidase. In this respect it is more similar to *T. brucei* proteinases such as trypanopain-Tb than to the trypsin-like enzyme called OP-Tb which only hydrolyses short peptides and not proteins (Troeberg *et al.*, 1996; Morty *et al.*, 1998).

3.5.1.3 Degradation of proteins

MCP-Tb (2 μg) completely degraded dephosphorlylated β-casein (51.3 μg) within a 90-min incubation period (Fig. 3.6A). The estimated specific activity of MCP-Tb was 17.1 mg dephospho-casein.mg MCP-Tb^{-1}.h^{-1}. The trypanosomal MCP-Tb activity against dephosphorylated β-casein is slightly greater than that reported for bovine MCP. MCP from bovine pituitary cells degrades dephosphorylated β-casein with a specific activity of 10-12 mg dephospho-casein.mg MCP.$^{-1}$ h^{-1} (Pereira *et al.*, 1992a). A six-fold greater concentration of MCP-rbc (12 μg; Fig. 3.6B) than MCP-Tb (2 μg) was required in the present study to completely degrade dephosphorylated β-casein (51.3 μg) within 90 minutes. The higher concentration of MCP-rbc is evidenced by visualisation of the enzyme bands in the 90-min digestion lane.

The specific activity of purified MCP-rbc was 2.85 mg dephospho-casein.mg MCP-rbc^{-1}.h^{-1} which is in agreement with caseinolytic specific activity of latent bovine pituitary MCP of 2-3 mg dephosphorylated casein degraded.mg MCP.$^{-1}$h^{-1} (Pereira *et al.*, 1992). The enhanced specific activity of the parasite MCP-Tb towards proteins such as casein may have a physiological significance in terms of degradation of host proteins to provide nutriment for the parasite or for rapid turnover of proteins in this highly active parasite.

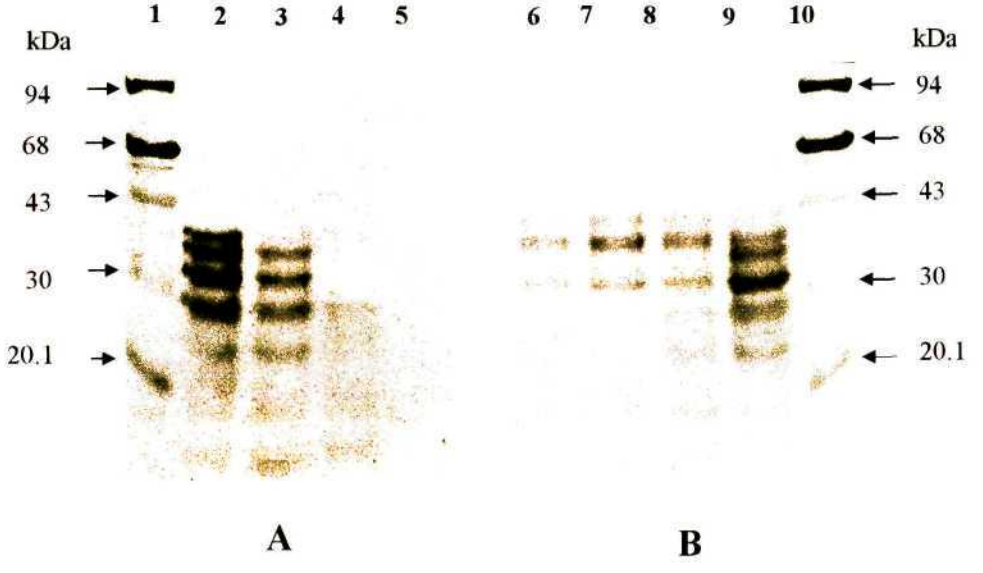

Figure 3.6. Activity of purified MCP-Tb and MCP-rbc against β-casein

Purified MCP-Tb (A) and MCP-rbc (B) were incubated with β-casein for different time periods at 37°C (Section 3.4.3.2). Proteolysis was terminated by boiling the reaction mixtures for 5 min, and degradation products were analysed by SDS-PAGE. Samples were: M_r markers as in Fig. 3.4 (lanes 1 and 10), reaction mixtures at 0 min (lanes 2 and 9), 45 min (lanes 3 and 8), 90 min (lanes 4 and 7) and 120 min (lanes 5 and 6). The degradation products were visualised on SDS-PAGE after silver staining.

3.5.2 Substrate Specificity

Evaluation of subsite specificity of the trypanosomal MCP-Tb cleavage of peptide substrates is shown in Table 3.2. The MCP-Tb manifests a broad specificity. It cleaves chymotrypsin-like bonds with bulky hydrophobic (Leu) or aromatic (Phe, Tyr) residues in position P_1 as well as polar residues with either basic (Arg) or acidic (Glu) residues. However, a potential substrate with a sulfur-containing residue (Met) in $P_1$was not readily hydrolysed, thus indicating that this site does have some discrimination. An oligopeptidase with trypsin-like activity described in *T. brucei* hydrolyses Z-Arg-Arg-AMC better than Z-Pro-Phe-Arg-AMC (Morty *et al.*, 1998). Conversely, MCP-Tb was found to prefer Z-Pro-Phe-Arg-AMC.

Comparisons of MCP-rbc and MCP-Tb activities show that specific peptidase activities of MCP-Tb were 1 to 5-fold higher than those of MCP-rbc for the different substrates tested (Table 3.2). The 'ratios' represent the magnitude by which specific activity of the MCP-

Tb exceeds that of MCP-rbc. The MCP-Tb showed an overal trend of a higher specific activity than that of the bovine enzyme. However, comparative data on specific activity of the MCP from bovine and trypanosome sources need to be treated with some caution. The purification protocols for the two enzymes differ. It is possible that the additional ammonium sulfate precipitation step involved in the purification of the erythrocyte MCP-rbc had effect on its specific activity.

Table 3.2. Hydrolysis of synthetic substrates by MCP purified from *T. brucei* and from bovine erythrocytes

Activity was assayed in the presence of a peptide-AMC (50 μM) or peptide-βNA (200 μM) substrate and proteasome (0.2 μg) under conditions described in Section 3.4.

Substrate	Proteasome activity ($nmol.min^{-1}.mg^{-1}$) MCP-Tb	MCP-rbc	MCP-Tb/MCP-rbc ratio
Chymotrypsin-like			
Z-Gly-Gly-Leu-AMC	16.20 ± 0.23	18.50 ± 0.70	0.88
Suc-Leu-Leu-Val-Tyr-AMC	10.85 ± 0.25	4.90 ± 0.19	2.21
Suc-Ala-Ala-Phe-AMC	8.79 ± 0.04	2.69 ± 0.08	3.27
H-Ala-Ala-Phe-AMC	7.77 ± 0.11	4.51 ± 0.30	1.72
Ac-Ala-Ala-Tyr-AMC	7.64 ± 0.42	2.82 ± 0.08	2.71
Suc-Leu-Tyr-AMC	7.55 ± 0.08	1.40 ± 0.05	5.39
Glut-Gly-Gly-Phe-AMC	3.15 ± 0.06	1.98 ± 0.06	1.59
H-Leu-AMC	0.40 ± 0.05	0.32 ± 0.05	1.25
Z-Gly-Ala-Met-AMC	0.20 ± 0.03	0.15 ± 0.01	1.33
Trypsin-like			
Z-Gly-Gly-Arg-AMC	2.40 ± 0.03	1.91 ± 0.04	1.26
H-Pro-Phe-Arg-AMC	1.73 ± 0.05	1.55 ± 0.06	1.12
Z-Phe-Arg-AMC	0.66 ± 0.01	0.60 ± 0.03	1.10
Z-Arg-Arg-AMC	0.47 ± 0.04	0.14 ± 0.01	3.36
Peptidyl glutamylpeptide hydrolase			
Z-Leu-Leu-Glu-βNA	0.72 ± 0.05	0.44 ± 0.04	1.64

Proteasomes have been shown to be sensitive to high salt concentration (Mason, 1990). Furthermore, salt precipitation is not beneficial for purification of an intact higher molecular mass 26S proteasome (Kanayama *et al.*, 1992; Dahlmann *et al.*, 1995). Be that as it may, the data shows the trypanosomal MCP-Tb hydrolyses Suc-Leu-Tyr-AMC and Suc-Leu-Leu-Val-Tyr-AMC considerably more rapidly than the bovine MCP-rbc. In contrast, Z-Gly-Gly-Leu-AMC was slightly more rapidly hydrolysed by the bovine MCP-

rbc than by the trypanosomal MCP-Tb. The specific activities of different proteasome preparations against different synthetic peptide substrates are compared in Table 3.3 with those obtained in this study for purified MCP-Tb and MCP-rbc.

Table 3.3. Peptidase specific activity of different proteasome preparations

Substrate	Enzyme source	Specific activity ($nmol.min^{-1}.mg^{-1}$)	Reference
Z-Gly-Gly-Leu-AMC	Bloodstream *T. brucei*	16.2	This study
	Bovine erythrocytes	18.5	This study
	Bovine erythrocytes	50.2	Chu-Ping *et al.*, 1994
	Bovine pituitary	146.7	Eleuteri *et al.*, 1997
	Bovine pituitary	120.0	Pereira *et al.*, 1992a
	Bovine spleen	53.3	Eleuteri *et al.*, 1997
	T. acidophilum	29.2	Tamura *et al.*, 1996
Suc-Leu-Leu-Val-Tyr-AMC	Procyclic *T. brucei*	3.9	Hua *et al.* 1996
	Bloodstream *T. brucei*	69.2	To & Wang, 1997
	Bloodstream *T. brucei*	6.3	Hua *et al.*, 1996
	Bloodstream *T. brucei*	10.9	This study
	Bovine erythrocytes	4.9	This study
	Bovine erythrocytes	10.2	Chu-Ping *et al.*, 1994
	Rat erythrocytes	9.4	Hua *et al.*, 1996
	Rabbit reticulocytes	116.2	Hough *et al.*, 1987
	Rat skeletal muscle	3.0	Dahlmann *et al.*, 1995
	Rat liver	1.9	Shibatani & Ward, 1995
	Xenopus spp oocyte	8.3	
	Murine EL4 thymoma	51.0	Yamada *et al.*, 1995
	T. acidophilum	23.7	Geier *et al.*, 1999
			Tamura *et al.*, 1996
Suc-Ala-Ala-Phe-AMC	Rat skeletal muscle	6.8	Dahlmann *et al.*, 1985
	Rat erythrocytes	3.1	Hua *et al.*, 1996
	Bovine erythrocytes	2.7	This study
	Bloodstream *T. brucei*	8.8	This study
	Bloodstream *T. brucei*	19.6	To & Wang, 1997
	Bloodstream *T. brucei*	2.0	Hua *et al.*, 1996
	Procyclic *T. brucei*	1.6	Hua *et al.*, 1996
	Procyclic *T. brucei*	6.2	To & Wang, 1997
	Murine EL4 thymoma	23.0	Geier *et al.*, 1999
H-Ala-Ala-Phe-AMC	Rat skeletal muscle	2.6	Dahlmann *et al.*, 1985

	Rat liver	3.9	Conconi *et al.*, 1998
	Bovine erthyrocytes	4.5	This study
	Bloodstream *T. brucei*	7.8	This study
	T. acidophilum	16.3	Tamura *et al.*, 1996
	Murine EL4 thymoma	12.0	Geier *at al.*, 1999
Suc-Leu-Tyr-AMC	Bovine erythrocytes	1.4	This study
	Bloodstream *T. brucei*	7.6	This study
	T. acidophilum	0.0	Tamura *et al.*, 1996
Glut-Gly-Gly-Phe-AMC	*T. brucei*	3.2	This study
	Bovine erythrocytes	2.0	This study
	Rat skeletal muscle	6.6	Dahlmann *et al.*, 1985
	Lobster muscle	0.2	Mykles & Haire, 1991
Z-Gly-Gly-Arg-AMC	Procyclic *T. brucei*	147.9	To & Wang, 1997
	Procyclic *T. brucei*	53.3	Hua *et al.*, 1996
	Bloodstream *T. brucei*	158.0	To & Wang, 1997
	Bloodstream *T. brucei*	27.9	Hua *et al.*, 1996
	Bloodstream *T. brucei*	2.4	This study
	Bovine erythrocytes	1.9	This study
	Rat erythrocytes	0.8	Hua *et al.*, 1996
	Rat skeletal muscle	31.2	Dahlmann *et al.*, 1985
	Human liver	3.3	Mason, 1990
	T. acidophilum	0.0	Tamura *et al.*, 1996
H-Pro-Phe-Arg-AMC	Procyclic *T. brucei*	43.3	To & Wang, 1997
	Procyclic *T. brucei*	10.0	Hua *et al.*, 1996
	Bloodstream *T. brucei*	13.1	Hua *et al.*, 1996
	Bloodstream *T. brucei*	34.2	To & Wang, 1997
	Bloodstream *T. brucei*	1.7	This study
	Bovine erythrocytes	1.6	This study
	Rat erythrocytes	2.8	Hua *et al.*, 1996
Z-Phe-Arg-AMC	Bloodstream *T. brucei*	0.7	This study
	Bovine erythrocytes	0.6	This study
	Rat skeletal muscle	0.0	Dahlmann *et al.*,1985
Z-Arg-Arg-AMC	Bloodstream *T. brucei*	0.5	This study
	Bovine erythrocytes	0.2	This study
	Rat skeletal muscle	7.4	Dahlmann *et al.*, 1985
Z-Leu-Leu-Glu-βNA	Bloodstream *T. brucei*	0.7	This study
	Bovine erythrocytes	0.4	This study
	Bovine erythrocytes	0.4	Chu-Ping *et al.*, 1994
	Bovine pituitary	480.0	Olowski *et al.*, 1993
	Bovine pituitary	135.0	Eleuteri *et al.*, 1997
	Bovine spleen	18.0	Eleuteri *et al.*, 1997

	Rat liver	12.6	Conconi *et al.*, 1998
	Rat liver	0.2	Shibatani & Ward,
	Rat skeletal muscle	75.0	1995
	Murine EL4 thymoma	12.0	Dahlmann *et al.*, 1995
	Lobster muscle	0.8	Geier *et al.*, 1999
			Mykles & Haire, 1991

Considerable variation can be observed in specific activity against similar substrates, depending on the cell type, isolation procedure or enzyme assay system used. While quite similar values were obtained in this study for MCP-Tb against Suc-Leu-Leu-Val-Tyr-AMC (10.9 nmol.min^{-1} .mg^{-1}) and that of Hua *et al.* (1996) of 6.3 nmol.min^{-1}.mg^{-1}, To and Wang (1997) reported a significantly higher value (69.2 nmol.min^{-1}.mg^{-1}).

In some cases major differences have been reported between different MCP preparations. For example, MCP isolated from *T. acidophilum* has no activity against Suc-Leu-Tyr-AMC (Tamura *et al.*, 1996), whereas the same substrate was found to be hydrolysed by both MCP-Tb and MCP-rbc in the present study. Also, Z-Gly-Gly-Arg-AMC-hydrolysing activity is present in MCP-Tb and MCP-rbc but absent in the archaebacterium proteasome (Tamura *et al.*, 1996). However, it is not possible to compare the present results on MCP-Tb activity against Z-Gly-Gly-Arg-AMC with those of similar enzymes isolated from bloodstream *T brucei* by other laboratories [*e.g.* 27.9 nmol.min^{-1}.mg^{-1} for Hua *et al.* (1996), or158 nmol.min^{-1}.mg^{-1}. for To and Wang (1997)] because their purification data is not available.

MCP-Tb is also shown to have PGPH activity as evidenced by Z-Leu-Leu-Glu-βNA hydrolysis. The specific activity of MCP-Tb or MCP-rbc against Z-Leu-Leu-Glu-βNA is consistent with that reported for MCP from bovine erythrocyte (Chu-Ping *et al.*, 1994) and lobster muscle (Mykles and Haire, 1991). However, the present PGPH results differ from the reported specific activity of MCP from rat skeletal muscle (Dahlmann *et al.*, 1995) or bovine pituitary (Olowski *et al.*, 1993; Eleuteri *et al.*, 1997). Overall, specific activities obtained in the present study compare well with those of earlier studies.

3.5.3 Susceptibility to Inhibitors

The sensitivity of the proteasome activities to inhibition by various typical serine protease inhibitors is shown in Table 3.4. Antipain, E-64 and EDTA were found to have no effect on the activity of either MCP-Tb or MCP-rbc (results not shown). The peptide aldehyde, Z-Leu-Leu-Leu-H, was the most potent inhibitor of both proteasomes tested, resulting in complete loss of activity against Z-Gly-Gly-Leu-AMC and Z-Gly-Gly-Arg-AMC. There was no significant difference in sensitivity between the trypanosomal and bovine proteasome to Z-Leu-Leu-Leu-H. Inhibition of the enzyme was dose dependent at inhibitor concentrations of 0 to 10 μM (Fig. 3.7). The IC_{50} values for MCP-Tb and MCP-rbc were 2 and 4 μM respectively.

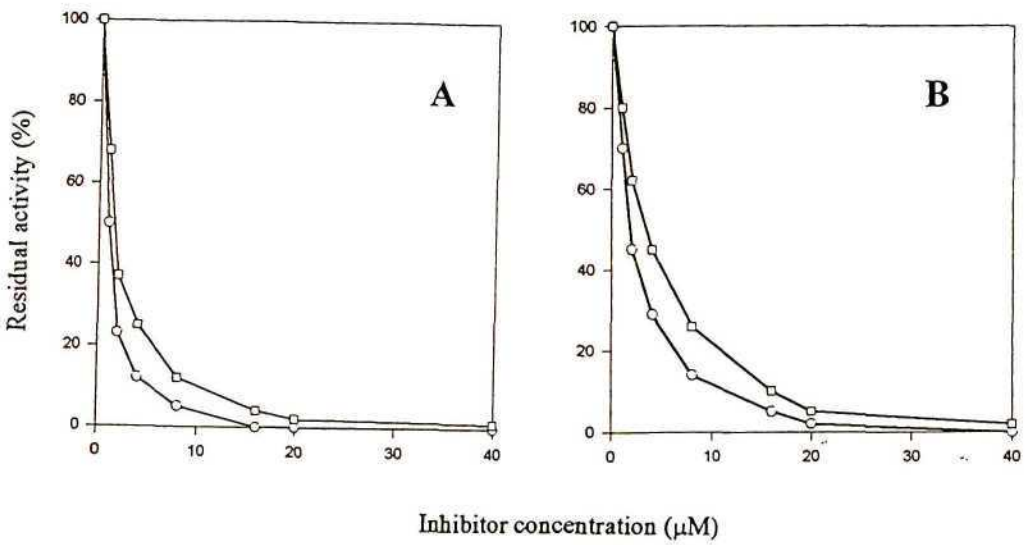

Figure 3.7. Concentration dependent inhibition of MCP-rbc and MCP-Tb by Z-Leu-Leu-Leu-H inhibitor

Purified bovine MCP-rbc (A), and MCP-Tb (B) were incubated with increasing concentrations of Z-Leu-Leu-Leu-H, for 30 min at 37°C followed by determination of the Z-Gly-Gly-Leu-AMC (O) and Z-Gly-Gly-Arg-AMC-hydrolysing activities (□). Values are expressed as % activity relative to control activities in samples containing no inhibitor.

There was also no significant difference between the IC_{50} values for the inhibition of the trypsin-like and chymotrypsin-like activities of MCP-rbc and MCP-Tb by Z-Leu-Leu-Leu-H. Although both Z-Gly-Gly-Arg-AMC and Z-Gly-Gly-Leu-AMC-hydrolysing activities of MCP-Tb were sensitive to Z-Leu-Leu-Leu-H inhibition, this peptide aldehyde could not

be used as an active site titrant for MCP-Tb in crude lysate from *T. brucei*. This is because the peptide aldehyde is known to inhibit other proteases especially calpains (Jensen *et al.*, 1995; Tsubuki *et al.*, 1996) and possibly OP-Tb (Morty *et al.*, 1998). The prohibitive cost of the proteasome specific inhibitor, lactacystin (Feteany *et al.*, 1995) obviated its use in the present study. Z-Leu-Leu-Leu-H was, however, useful for inhibition studies on the purified proteasome.

Chymostatin inhibited Z-Gly-Gly-Leu-AMC activity of MCP-Tb and MCP-rbc by 70% whereas their Z-Gly-Gly-Arg-AMC activity was inhibited by 30% (Table 3.4). Leupeptin inhibited trypanosomal MCP-Tb and bovine MCP-rbc activity against Z-Gly-Gly-Arg-AMC to a similar extent (82% and 75%, respectively). The trypsin-like activity of the parasite proteasome appears to be slightly more sensitive to leupeptin inhibition than that of the bovine proteasome.

Table 3.4. Effect of various serine protease inhibitors on the activity of MCP-Tb and MCP-rbc
Purified MCP preparations were incubated for 30 min at 25°C with inhibitors in assay buffer as described in Section 3.4.4. The enzyme activity was determined at 37°C against Z-Gly-Gly-Leu-AMC or Z-Gly-Gly-Arg-AMC. Values are expressed as percentage relative enzyme activity of the control enzyme (without inhibitor) for each substrate.

		Relative activity (%)			
		Z-Gly-Gly-Leu-AMC		Z-Gly-Gly-Arg-AMC	
Inhibitor	Conc. (μM)	MCP-Tb	MCP-rbc	MCP-Tb	MCP-rbc
Z-Leu-Leu-Leu-H	10	0	3	2	7
Chymostatin	50	32	28	71	69
Leupeptin	50	65	79	18	25
AEBSF	300	84	87	68	73
N-Tosyl-Lys-CH_2Cl	250	116	98	85	95
N-Tosyl-Phe-CH_2Cl	250	85	92	95	88
Benzamidine	1000	86	91	77	83
α-Chymotrypsin-trypsin Inhibitor	50	87	93	91	102

The patterns of inhibition of MCP-Tb and MCP-rbc by the various inhibitors are consistent with their different activities. However, the degree of inhibition does depend on the specific activity of the purified enzyme and the concentration of the inhibitor used. For example, it has been shown that 2 mM chymostatin is required to inhibit Suc-Leu-leu-Val-Tyr-AMC hydrolysing activity completely in the 20S proteasome from *T. acidophilum,* whereas only 0.25 mM inhibits rabbit reticulocyte-MCP activity. Leupeptin (1 mM) inhibited the Suc-Leu-Val-Tyr-AMC activity by 40% (Ditzel *et al.*, 1997) whereas 0.25mM leupeptin achieved a 12% inhibition of the same substrate (Hough *et al.*, 1987).

AEBSF, which is known to inhibit a wide range of serine proteinases, had minimal effect on the activities of the proteasomes. There was a 16 and 13% inhibition of chymotrypsin-like activity for *T. brucei* and bovine-derived proteasome, respectively. The trypsin-like activity was apparently more sensitive to AEBSF with 32% and 27% inhibition of activity for trypanosomal and bovine proteasome respectively. In bovine pituitary MCP, AEBSF (1 mM) show minimal inhibition of 5% of chymotrypsin-like activity (Orlowski *et al.*, 1993).

TLCK and TPCK also had minimal effects on the trypsin-like and chymotrypsin-like activities of the proteasomes. Chymotrypsin-like activity was even partially stimulated by TLCK, which is consistent with the previous observations on *T. brucei* and rabbit reticulocyte proteasomes (Hough *et al.*, 1987; Hua *et al.*, 1996). Benzamidine and α-chymotrypsin-trypsin inhibitor had no significant effect on the proteasome activities even at high concentrations. In archaebacterium 20S proteasome, benzamidine (10 mM) was even shown to stimulate chymotrypsin-like activity (Ditzel *et al.*, 1997).

3.5.4 Effect of pH on MCP-Tb activities

The effect of pH on the activities towards Z-Gly-Gly-Leu-AMC, Z-Gly-Gly-Arg-AMC and Z-Leu-Leu-Glu-βNA is shown in Fig. 3.8. The enzyme was stable over a broad range of pH-values between 6 and 10. No residual activity remained at below pH 4. Half-maximal activity remained at pH 5.0. The chymotrypsin-like and PGPH activities had similar pH profiles with optimum activity for hydrolysis of Z-Gly-Gly-Leu-AMC and Z-Leu-Leu-Glu-βNA being 7.5 while the activity towards Z-Gly-Gly-Arg-AMC was maximum at about pH 11.0

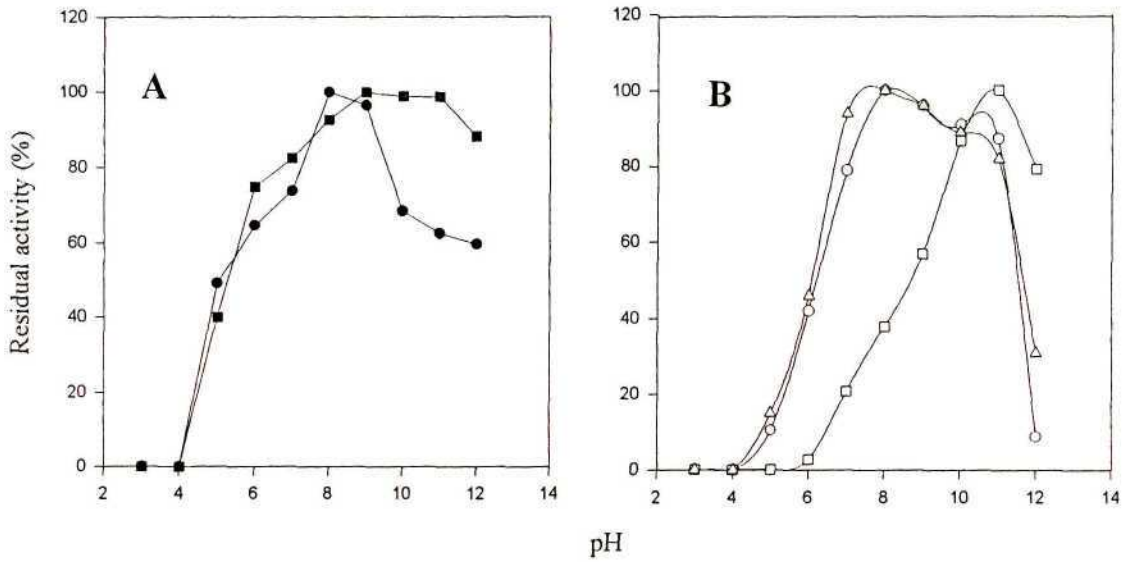

Figure. 3.8. The effect of pH on the three activities of MCP-Tb

The pH stability (A) was determined by incubating the enzyme for 30 min at a range of pH values from 3 to 12. The residual enzyme activity against Z-Gly-Gly-Leu-AMC (●) and Z-Gly-Gly-Arg-AMC (■) was assayed at pH 8.0 as described in Section 3.4.5.2. The pH-activity profile (B) against Z-Gly-Gly-Arg-AMC (□), Z-Gly-Gly-Leu-AMC (O) and Z-Leu-Leu-Glu-βNA (Δ) were performed over the pH range 3-12 as described in Section 3.4.5.2, except that assay buffer was replaced by Acetate/Mes/Tris buffers. Values are expressed as a percentage of maximal activity recorded for each substrate.

3.6 Discussion

Proteases of parasitic protozoa have been implicated in a wide range of host-parasite interactions including the invasion of host cells, processing of host proteins for nutrition, evasion of host immune responses, and transformation of parasites from one developmental stage to another (North, 1991; Lonsdale-Eccles *et al.*, 1993; McKerrow *et al.*, 1993). Cysteine proteases are found in *T. brucei* (Lonsdale-Eccles and Mpimbaza, 1986, Troeberg *et al.*, 1996), *T. congolense* (Mbawa *et al.*, 1991b), *T. cruzi* (Murta *et al.*, 1990), and *Leishmania* spp. (Coombs *et al.*, 1991), where they are mainly responsible for protein degradation within lysosomes. A cytosolic serine protease has been identified in *T. brucei* (Kornblatt *et al.*, 1992, Morty *et al.*, 1998) and membrane-bound metalloproteases are present in *T. brucei* (El-Sayed and Donelson, 1997), *Leishmania* spp (Etges and Bouvier, 1993) and *T. cruzi* (Bontempi *et al.*, 1984). Although a multicatalytic protease (proteasome) has been detected in *T. cruzi* (Gonzalez *et al.*, 1996) and *T. brucei* (Hua *et*

al., 1996; To and Wang, 1997; Mutomba *et al.*, 1997; Lomo *et al.*, 1997), biochemical characterisation and contribution of the parasite MCP to pathogenicity remains unclear.

In this chapter the author reported an effective purification procedure for MCP from bloodstream *T. brucei* and presented a biochemical comparison between the parasite protease (MCP-Tb) and the bovine host proteasome (MCP-rbc) activities. Different protocols involving column chromatography have been used to purify the proteasome from different tissues (Chu-Ping *et al.*, 1992; Dahlmann *et al.*, 1995; Hua *et al.*, 1996; Orlowski *et al.*, 1997). Ammonium sulfate precipitation as a first step in a purification protocol is common to those protocols where starting material is not limiting. However, where starting material is limited, as in the case of cultured cells, this step has usually been avoided. At the start of this study, no procedure had been described for the isolation of proteasomes from African trypanosomes. Subsequently, however, the isolation of proteasomes from *T. brucei* (Hua *et al.*, 1996) has been described. This protocol differs from that described in the present study. For instance, Hua *et al.* (1996) employed a combination of anion exchange chromatography and a series of glycerol gradient sedimentation steps in an attempt to isolate the proteasome. However, reports (Chu-Ping *et al.*, 1994; DeMartino *et al.*, 1994) indicate that a 700 kDa proteasome activator (and probably other high molecular mass proteins) co-purified with the catalytic 20S complex on an anion exchange column under the stated conditions (Hua *et al.*, 1996). It is unlikely that proteins of similar molecular masses such as the 20S proteasome and its 700 kDa activator may be separated by stepwise density gradient procedures but the presence of such associated (contaminating) proteins may not be revealed on non-denaturing polyacrylamide gels. It is not yet known whether the 700 kDa activator protein is present in trypanosomes. Nevertheless, the lack of demonstration of the activator protein may not be considered as proof for the absence of such a protein. Other possible contaminants are high molecular mass proteases, tricorn protease (Tamura *et al.*, 1996), or a giant protease (Geier *et al.*, 1999) recently described in eukaryotic cells but yet to be identified in parasitic protozoa. The data on purification of MCP from *T. brucei* (Hua *et al.*, 1996) was not provided and as such it is not possible to compare their yield with that obtained with the present protocol.

Although several reports show that the relative molecular mass of the 20S proteasome from archaebacterium and eukaryotic cells is 700 kDa (Coux *et al.*, 1996; Tanaka 1998) the relative molecular mass of MCP-Tb was estimated in the present study to be 590 kDa

by gradient PAGE. The MCP-Tb was found (on a reducing SDS-PAGE) to have components of at least four different molecular masses; 27, 26, 22 and 21 kDa. If equal amounts of the four components are assumed, approximately six of each would be needed to make up the total complex. This argument implies that MCP-Tb, like the *Thermoplasma* and yeast 20S proteasomes, is composed of a stack of four rings of subunits (Löwe *et al.*, 1995; Groll *et al.*, 1997). However, the MCP-Tb differs from *Thermoplasma* and yeast proteasomes in having six, instead of seven, subunits forming each ring of the complex (Löwe *et al.*, 1995; Seemuller *et al.*, 1995; Groll *et al.*, 1997) indicating that the parasite MCP-Tb might have lost four of its MCP-Tb subunits in the course of evolution. However, a reduction in the number of proteasomal subunits has not been reported in other systems although the immunomodulatory cytokine, γ-interferon (γ-IFN) does induce replacement of three pairs of homologous proteasomal subunits (X, Y and Z, by LMP7, LMP2 and MECL1, respectively) in eukaryotic cells (Nandi *et al.*, 1996). This causes an alteration of the proteolytic specificity to favour antigen presentation (Golgberg *et al.*, 1997). This ability to switch indicates that some of the subunits of a proteasome may be relatively easily exchanged and so may be loosely bound together. This situation may also apply to MCP-Tb because different estimates of the M_r were obtained in the present study depending upon the estimation technique. In gel electrophoresis (a fairly harsh procedure) a relatively smaller value (Mr 590 000) was obtained than when the gentle procedure of gel filtration through Sephacryl S-300 (M_r 670 000) was used. Furthermore, the size of 590 kDa was estimated on a PAGE gel which separates proteins on the basis of their charge to mass ratios, resulting in inaccurate M_r determinations. If the same assumptions are made about the subunit masses as was done above, MCP-Tb may also be composed of seven, rather than six, subunit domains with a calculated M_r of 672 000. Taken together, the results suggest that MCP-Tb may also have readily exchangeable domains and this, in turn, may explain why different preparations of the *T. brucei* proteasome have different enzymatic properties.

In the present study it was shown that MCP-Tb has a chymotrypsin-like activity that hydrolyses substrates faster than the trypsin-like activity and a low PGPH activity. Thus, it is similar to the enzyme from eukaryotic organisms and unlike that from the archeabacterium *Thermoplasma* 20S proteasome (Tamura *et al*, 1996). This observation corroborates earlier reports (Hua *et al.*, 1996; To and Wang, 1997) on a proteasome isolated from *T. brucei*. Characterisation of subsite specificity indicates that MCP-Tb

catalyses cleavage of a wide range of substrates with different amino acid residues in P_1 position (Leu, Phe, Tyr, Arg, Glu; Table 3.2). These may be distinguished as aromatic (Phe, Tyr), large (bulky) hydrophobic residues (Leu, Val) and small neutral residues (Gly, Ala). If Met occupies the P_1-binding position, the cleavage efficiency diminishes.

The results also demonstrate the importance of secondary binding site interactions. Thus, a tripeptide (e.g. H-Pro-Phe-Arg-AMC) was hydrolysed much more readily than a dipeptide (e.g. Z-Phe-Arg-AMC). In addition, whereas it may be assumed that Leu in position P_1 (e.g. Z-Gly-Gly-Leu-AMC) favours hydrolysis, the influence which is simultaneously exerted by residues in positions P_2, P_3 and P_4 that may contribute to substrate binding should also be taken into account. The faster hydrolysis of Suc-Leu-Leu-Val-Tyr-AMC than Suc-Leu-Try-AMC by MCP-Tb suggests that some residues play complementary roles in specificity. This is in agreement with the observation that the nature of amino acid residue in the P_3 position directs substrates to distinct catalytic sites of the mammalian proteasome (Cardozo *et al.*, 1994). Indeed, it has been proposed that the residue in the P_1 position does not contribute to the selection of a cleavage site by the proteasome, but rather that the residues in the P_3 and P_4 positions determine cleavage specificities of the proteasome (Cardozo *et al.*, 1994; Tsubuki *et al.*, 1996). Furthermore, it appears that the residue in the P_2 position is solvent-exposed, unconstrained by the active site cleft, and therefore also unlikely to contribute to the cleavage specificity (Groll *et al.*, 1997).

Although fluorogenic peptides have been useful for the characterisation of the different proteolytic activities, it is noteworthy that they have little relevance for the cleavage specificities in non-synthetic peptides or proteins. An analysis of degradation products of oxidized insulin B chain by purified human 20S proteasomes has shown that most peptide bonds can be cleaved by the proteasome and many of the preferred sites cannot be described in terms of the specificities described by experiments with fluorogenic peptides (Ehring *et al.*, 1996). The same is true for the cleavage pattern observed in ovalbumin (Dick *et al.*, 1994), gonadotropin-releasing hormone (Leibovitz *et al.*, 1995), bradykinin (Zolfaghari *et al.*, 1987) and neurotensin (Cardozo *et al.*, 1992). However, just like MCP-rbc, on the basis of multiple preferred substrates and reactions with different class-specific protease inhibitors, MCP-Tb still qualifies to be described as having the classical multiple peptidase activities, designated chymotrypsin-like, trypsin-like and PGPH activities (Cardozo, 1993). Indeed we demonstrated for the first time in the present study that

caseinolytic activity of MCP-Tb differs from that of MCP-rbc in terms of specific activity for the hydrolysis of dephosphorylated β-casein.

Studies on trypanosomatids show that class-specific inhibitors can kill parasites *in vitro* (Rosenthal *et al.*, 1991; Mutomba *et al.*, 1997). For this reason, the inhibitory effects of various serine protease inhibitors on the activities of MCP-Tb and MCP-rbc were examined (Table 3.4). Peptide aldehyde Z-Leu-Leu-Leu-H exhibited considerable inhibitory effects on the proteasome consistent with earlier reports on the proteasome from bovine brain and lungs (Jensen *et al.*, 1995; Tsubuki *et al.*, 1996). Similar inhibition of MCP-Tb and MCP-rbc may demonstrate a close relationship between the structures of the distantly related enzymes. This close relationship suggests that the catalytic mechanism of the MCP-Tb activity may be strictly conserved (Lupas and Baumeister, 1998). On the other hand, the observed lack of specificity of Z-Leu-Leu-Leu-H to MCP-Tb implies that a drug design based on this peptide inhibitor may not achieve the required selective toxicity in therapy.

Neither the trypsin- nor chymotrypsin-like activities of MCP-Tb or MCP-rbc were inhibited by the classical chymotrypsin-like and trypsin-like inhibitors, TLCK or TPCK, thus indicating that these proteasomes do not have the required residues near the active site for the reagents to alkylate, notably His or Ser. On the other hand, inhibition of MCP-Tb by leupeptin and chymostatin indicates that a tetrahedral intermediate is involved in the catalysis reaction. Inhibition of the trypsin-like activity was most effective with leupeptin, while chymostatin was less effective. Conversely, chymostatin inhibited the chymotryptic-like activity more effectively than the trypsin-like activity, which was scarcely inhibited by chymostatin. This suggests that the two activities of MCP-Tb are distinct, as is the case in MCP-rbc (Stein *et al.*, 1996).

Unfortunately, the multiple activities of the proteasome present a problem in kinetic analysis of individual activities, because of an overlap in substrate specificity (Stein *et al.*, 1996. Notably, the apparently better substrate for chymotrypsin-like activity may simultaneously be hydrolysed by the trypsin-like activity of the same proteasome. The possibility of the selective inhibition of chymotrypsin-like or trypsin-like activities of MCP-Tb by the alternate use of chymostatin and leupeptin is therefore of interest for the characterisation of MCP-Tb. The slight inhibition by chymostatin of the trypsin-like

activity may be ascribed to steric hindrance of the binding of the Z-Gly-Gly-Arg-AMC substrate. The peptide aldehyde inhibitors are structural analogues of the peptide substrates and compete for the substrate binding sites.

The pH activity and stability studies suggest that the trypanosomal proteasome is localised in an alkaline, hence non-lysosomal, compartment as reported in other eukaryotic cells (Tanaka *et al.*, 1989; Rivett *et al.*, 1992; Peters *et al.*, 1994). The trypanosomal proteasome exhibited a pH optimum that is too high for the proteasome to function efficiently in the mostly acidic environment of the lysosomes. Indeed, the purified enzyme lost its activity completely at a pH less than 4.0. This was contrary to the stability at pH 2.5 for a similar large multicatalytic protease from mammalian cells (Tsuji and Kurachi, 1989). The pH optimum of 11 for the trypanosomal proteasome activity against Z-Gly-Gly-Arg-AMC also differs from that reported by Mason (1990), who showed that human multicatalytic protease hydrolyses the same substrate with a pH optimum of 10.0. There is, however, a consensus on the pH optimum of about 7.5 for the chymotrypsin-like activity in the human multicatalytic protease (Mason, 1990), rat enzyme (Dahlmann *et al.*, 1985) and hamster kidney cells (Tsuji and Kurachi, 1989). Distinct pH-activity profiles imply different activities of the same enzyme rather than the same activity hydrolysing different substrates in a non-specific manner.

Zymogram analysis of MCP-Tb activity (Fig 3.5), as well as *in vitro* degradation of proteins (Fig. 3.6), indicate that the MCP-Tb hydrolyses whole proteins. Thus, MCP-Tb can readily be distinguished from another trypsin-like, cytosolic hydrolase called OP-Tb (oligopeptidase from *T. brucei*; Morty *et al.*, 1998) that hydrolyses only short peptides and not proteins (Troeberg *et al.*, 1996). Since proteasomes degrade protein substrates into peptides of focused length (mostly between 4 and 10 residues), a property shared between prokaryotic and eukaryotic proteasomes (Wenzel *et al.*, 1994; Ehring *et al.*, 1996), it seems likely that OP-Tb, possibly in conjunction with other cytosolic peptidases, may complete the degradation initiated by MCP-Tb by degrading the peptides generated by the proteasome complex. Thus, MCP-Tb and OP-Tb may play complementary roles in extralysosomal protein and polypeptide degradation within *T. brucei*.

The new purification protocol for the isolation of MCP-Tb from *T. brucei* developed in this study (Lomo *et al.*, 1997) should be applicable to the isolation of similar enzymes from

other protozoan organisms. Further, the results presented in this chapter show that MCP-Tb has substrate specificity and inhibitor profile characteristics that identifies MCP-Tb as a proteasome. Curiously, MCP-Tb was found to have a relative molecular mass that was less than that of the MCP-rbc. To date, there has been no report on M_r differences between 20S proteasomes isolated from different cell-types. Consequently, structural and immunochemical studies on MCP-Tb and MCP-rbc are discussed in Chapter 4.

Chapter 4

Structural and immunochemical studies of MCP-Tb

4.1 Introduction

The structures of 20S proteasomes from *Thermoplasma* and eukaryotes are virtually indistinguishable on electron micrographs (Dahlmann *et al.*, 1989, Baumeister *et al.*, 1997). Projections down the cylinder axis (end-on views) show a ring-shaped structure, whereas projections perpendicular to the cylinder axis (side-on views) appear rectangular with a characteristic pattern of four striations, suggesting a stack of four rings (Löwe *et al.*, 1995). An immunoelectron microscopic investigation has shown that the α-subunits form the outer rings while the β-subunits forms the inner rings (Grziwa *et al.*, 1991). Mass measurement by means of STEM (Scanning Transmission Electron Microscopy) has helped to determine the stoichiometry ($\alpha_7\beta_7\beta_7\alpha_7$) and metal decoration studies revealed the sevenfold symmetry of the complex (Pühler *et al.*, 1992). The significant implication of the symmetry is that a maximum of fourteen different subunits (seven α-type and seven β-type) can be accommodated in a single proteasome.

Although the quaternary structure of the eukaryotic complex is very similar to that of the *Thermoplasma* proteasome, there are variations reported in the literature with respect to the dimensions of the complex (Arrigo *et al.*, 1988, Pühler *at al.*, 1992, Baumeister *at al.*, 1997). Furthermore, the present study shows that MCP-Tb has a lower relative molecular mass compared to the bovine MCP-rbc. Consequently, MCP-Tb was subjected to structural investigation by electron microscopy of negatively stained particles. Further investigation involved determination of N-terminal amino acid sequences of a protein component of MCP-Tb. The protein sequence information was used to identify MCP-Tb as a proteasome complex, and to determine phylogenetic relationship of the MCP-Tb subunit and its homologues which are part of proteasomes from other organisms.

Antibodies were raised against MCP-Tb, VSG and PA700 for use in immunochemical and immunolocalisation studies and inhibition of proteasome activity. Polyclonal, monoclonal and

anti-peptide antibodies constitute the major types of antibody that can be produced. Knowledge of pitfalls associated with each type of antibody may assist in the choice of the type of antibody and interpretation of otherwise unexpected results. For this reason, types and characteristics of the various antibody groups will be discussed briefly with regard to the experimental requirements of this study.

Polyclonal antibodies are raised by immunization of experimental animals with purified antigens. Preparations of these antibodies from serum contain a population of antibodies that react with a number of epitopes within the inoculated target protein. Monoclonal antibodies on the other hand are the homogeneous products of a single B-cell clone targeting specificity for a particular epitope (Köhler and Milstein, 1975). Monoclonal antibodies are generated by fusing a single primed B-cell with an immortal tumour cell to produce an immortal B-cell hybridoma. Anti-peptide antibodies are raised against sequences within the primary sequences of a precursor or mature proteins. Careful selection of the peptide often results in anti-peptide antibodies that recognise the whole parent protein.

The polyclonal nature of an antibody preparation may be an advantage as tissue or cell fixation for immunolocalisation often results in progressive destruction or cross-linking of one or more target epitopes. For this study, immunization with purified proteins was considered suitable since the polyclonal antibody recognizes a number of targeted epitopes, and theoretically such an antibody preparation should have a higher chance of producing successful labeling by recognizing at least one surviving epitope in a fixed antigen. This is particularly important if the target antigen is sensitive to the tissue fixation and processing regime which precedes tissue sectioning and immunolabeling.

However, the inherent heterogeneity of polyclonal antibodies may also pose several problems precluding, for example, the exclusive recognition of a single specific epitope (e.g. the active site of an enzyme). In addition, 'background' or 'contaminating' antibodies may arise from highly immunogenic trace contaminants in the antigen preparation, eliciting a stronger or rivaling antibody response to that elicited by the major antigen present in the inoculated preparation. The latter problem may be assessed in part by determining the specificity of labeling, by probing a western blot of a crude homogenate of tissue (i.e. from which the

antigen used to raise the antibody, was isolated). In the present study, the antibodies targeted only the protein against which it was raised. Such results minimised the likelihood that the antibody preparation targeted common epitopes in other proteins. Hence, the polyclonal antibodies raised were used for labeling without the need to affinity purify the antibody fractions. Affinity purification has a potential disadvantage, as it tends to select for the lower affinity antibodies as the more avidly bound antibodies may be too tightly bound to the immobilized target antigen to be easily eluted in a non-denatured form.

Monoclonal antibodies usually give a strong reaction, provided that the epitope is not cross-linked or destroyed by fixation. Monoclonal antibodies have the potential to recognize common epitopes in other proteins and are expensive, but because they target a single epitope, they are usually high titre and give a strong signal. For reasons of costs, and considering that monoclonal antibodies target a single epitope that may not survive fixation, monoclonal antibodies were not used in the present study.

Anti-peptide antibodies constitute a unique reagent for which, as yet, there is no substitute (Briand *et al.*, 1985). The peptide antibody approach provides one of the few ways in which protein and antigen processing may be studied. Such antibodies may be used to circumvent the cross-reactivity between proteins such as cathepsins L and H that have conserved sequences in some regions (Ritonja *et al.*, 1988). The amino acid sequence of MCP-Tb was not available and studies involving the use of anti-peptide antibodies were therefore not possible in the present study.

The antibodies used in the immunochemical and immunolocalisation studies reported in this thesis were produced against proteins purified during the course of the study. The purified proteins included VSG (Section 4.4), 20S proteasome (MCP-Tb) from *T. brucei* (Section 3.3.1), and PA700 from bovine red blood cells (Section 6.2). Antibodies raised against the MCP-Tb were used for immunolocalisation of the enzyme in *T. brucei* sections (Chapter 5), and to test for antigenic similarities between the parasite MCP-Tb and the 20S proteasome (MCP-rbc) from bovine red blood cells (Section 3.3.2). The ability of these antibodies to inhibit proteasome activity was also investigated (Chapter 4). Anti-VSG antibodies were used in immunolocalisation studies (Chapter 5), while anti-PA700 antibodies were used to study the

interaction between MCP-Tb and the bovine PA700 activator, reported in Chapter 6. For reasons of ease of presentation and flow of results, the characterisation of anti-VSG and anti-PA700 antibodies is shown here in Chapter 4 with that of the other antibodies.

4.2 Electron microscopy

To examine the morphology of MCP, a drop of purified MCP-Tb or MCP-rbc (0.1 mg protein/ml) was placed on a Formvar coated nickel grid. The sample was dried by absorbing the excess solution with filter paper placed by the sides of the grids. The grids were stained in the dark at 25°C for 10 min with 1% (m/v) aqueous uranyl acetate, and then for 10 min with alkaline lead citrate. The protein samples were examined under a transmission electron microscope Philips CM 120 Biotwin at magnifications 74 000 and 105 000.

4.3 Protein sequencing

Two-dimensional electrophoresis of MCP-Tb or MCP-rbc (15 μg) was performed as described in Section 2.9.1. The separated protein components were electrophoretically transferred to a PVDF membrane and stained with Coomassie Blue (see Section 2.10.1). The membrane region containing the selected protein spot was cut out with a clean razor. Protein (20 pmol) was sequenced on an automated Perkin Elmer Applied Biosystems Procise 491 (Molecular Biology Unit, University of Natal). Phenylthiohydantoin (PTH) amino acids were identified online with a 785A Applied Biosystems PTH analyser by reversed-phase HPLC. A "FASTA Search" of the Swiss-Prot database was conducted for sequence similarities. The N-terminal amino acid sequences of various α- and β-subunits of eukaryotic proteasomes were aligned with "DIALIGN"(Morgenstern *et al.*, 1996).

4.4 Purification of VSG

Variant surface glycoproteins were purified from *T. brucei* using a technique based on extraction of acid treated proteins into chloroform/methanol, followed by selective repartition into an aqueous salt solution (Jackson *et al.* 1985).

4.4.1 Reagents

Tes buffer, pH 7.5 [20mM Tes, 140 mM NaCl, 5mM $MgSO_4$, 0.1 mM EGTA, 0.1 mM PMSF, 10 mM glucose and 75 mM benzyl alcohol]. Tes (4.584 g), NaCl (8.182 g), $MgSO_4.7H_2O$

(1.233 g), (EGTA 0.038 g), glucose (1.802 g) and benzyl alcohol (7.98 ml) were dissolved in 800 ml of dist. H_2O, adjusted to pH 7.5 with NaOH and made up to 1 litre.

4.4.2 Procedure

A suspension of bloodstream form of *T. brucei* (approx. 1 x 10^9 cells) in 10 ml of PSG buffer, pH 7.5 at 0°C was added with stirring to an equal volume of Tes buffer, pH 7.5 so that the final concentration of cells was 5x10^8 cells/ml. The mixture was incubated at 37°C for 5 min then an equal volume of Tes buffer without benzyl alcohol at 0°C added and centrifuged (12 000 x g; 5 sec). The supernatant was carefully removed and concentrated 10-fold by dialysing against PEG 20 kDa at 4°C. The protein in the concentrate was further purified with 0.2 volumes of 50% trichloroacetic acid and centrifuged (3 000 x g, 10 sec). The precipitated protein was resuspended in distilled deionized water (4 ml). The suspension was extracted with 20 volumes of chloroform/methanol (2:1, v/v) with vigorous shaking for 5 min and then stored overnight at 5°C. This storage procedure increases the final yield of purified VSG.

The extract was separated into two phases by centrifugation (12 000 x g; 1h) after the addition of 0.2 volume of 0.9% (w/v) NaCl solution. The upper aqueous phase contained pure VSG and was removed by aspiration and dialyzed against a large volume of distilled deionized water, at 4°C for a total of 36 h. The purity of VSGs was assessed by SDS-PAGE (Laemmli 1970) as outlined in Section 2.6.

4.5 Production of antibodies against VSG, MCP-Tb and PA700 in chickens

Polyclonal antibodies were raised in chickens against purified VSG (Section 4.4), MCP-Tb (Section 3.3.1) and bovine proteasome activator PA700 (Section 6.2) as outlined in Section 2.10.

4.6 ELISA and western blotting analysis of proteins

An ELISA was performed on each antibody as outlined in Section 2.12. Immunoblot analyses were carried out as outlined in Section 2.11. The specificity of the antibodies was tested on blots of purified antigen alongside a crude trypanosome lysate. The crude lysates (high speed supernatant) were prepared by lysis of *T. brucei* cells, followed by centrifugation of the

homogenate (100 000 x g, 4°C; 1 h) as outlined in Section 3.3.1. An aliquot (5 µl) of the supernatant was subjected to either native PAGE (3 to 10% acrylamide gradient) or SDS-PAGE (10%). The gel-separated proteins were transferred to nitrocellulose membranes as described in Section 2.11.2. The different chicken IgY preparations, at concentrations determined from the ELISA above, were used to probe the western blots.

4.7 Inhibition of MCP activity by antibodies

Assays for inhibition of MCP activity by antibodies were carried out as described for cathepsin L (Coetzer *et al.*, 1991). MCP-Tb or MCP-rbc (0.1 µg) were incubated at 37°C for 15 min with chicken anti-MCP IgY, or pre-immune chicken IgY, at different antibody concentrations. After incubation, the enzymic reactions were started by the addition of the substrate Z-Gly-Gly-Leu-AMC (25 µM). The released fluorophore was measured fluorometrically in a continuous assay as outlined in Section 3.4.1. Inhibition of the enzyme activity by anti-MCP antibodies was expressed as a percentage of the activity in the presence of pre-immune IgY.

4.8 Results

4.8.1 Electron microscopy

Examination of negatively stained MCP-Tb and MCP-rbc particles under the electron microscope revealed both similarities and differences (Fig. 4.1A and Fig. 4.1B). Like MCP-rbc, MCP-Tb assumes a barrel-shape with a rectangular side view (14.5 ± 0.3 nm long and 10 ± 0.5 nm wide) with a pseudo 7-fold symmetry. The end-on view is donut-ring like with an estimated outer diameter of 10 ± 0.3 nm and a dark stained inner diameter of 2.9 ± 0.4 nm. There was no significant difference in the monomeric dimensions of MCP-Tb and MCP-rbc donut-like structures. MCP-Tb differs from MCP-rbc in that MCP-Tb forms a peculiar "end-on" association of particles to create long ribbon-like chains [up to 200 nm long (Fig. 4.1A)]. The chains of MCP-Tb units do not break upon dilution of the sample (4.1C) implying strong interactions between the associated units. There was no obvious association between MCP-rbc units even at high concentration of the enzyme (Fig. 4.1B) or when the enzyme was diluted (Fig. 4.1D)

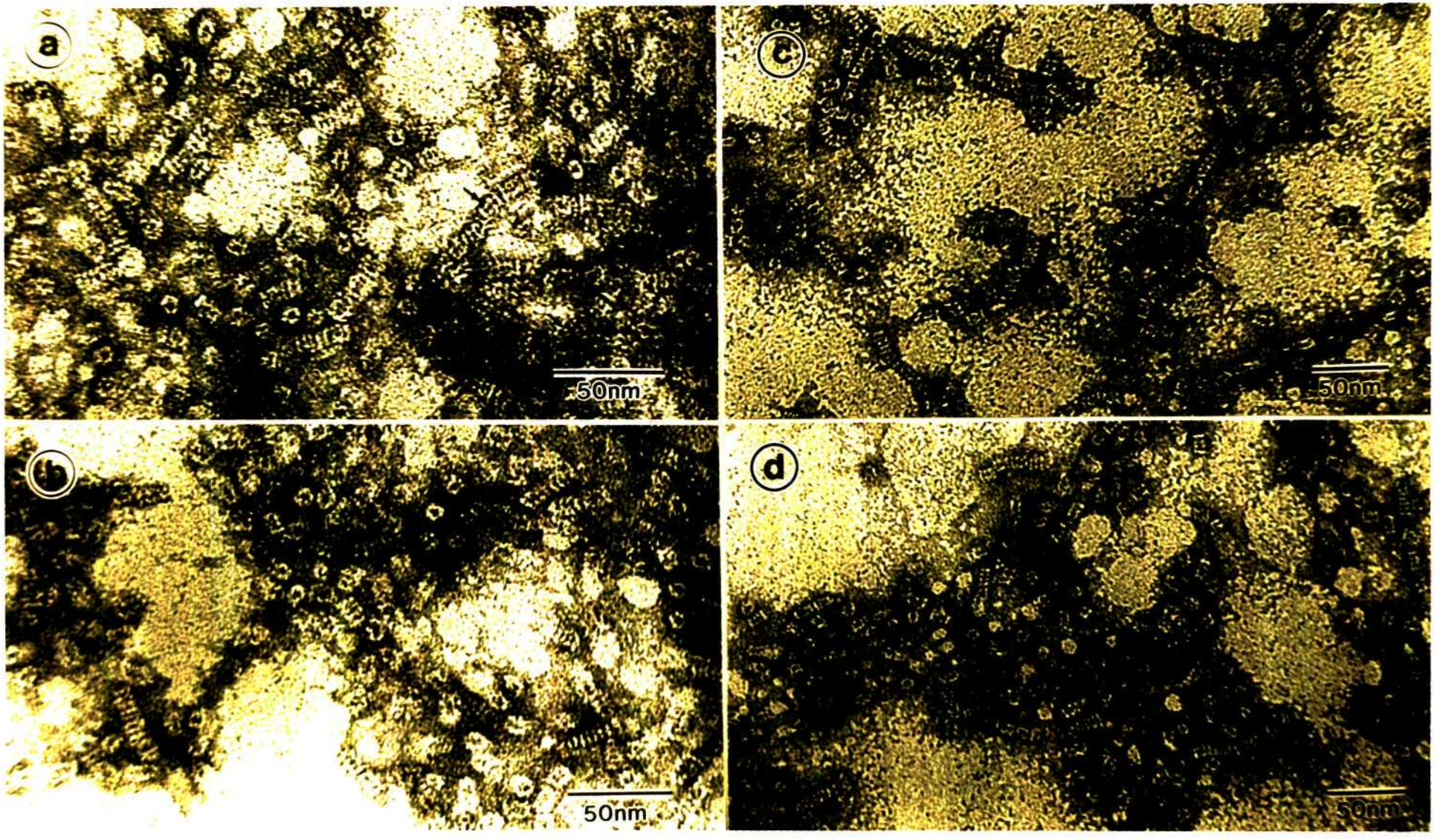
a
50nm
c
50nm
b
50nm
d
50nm

Figure 4.1 Electron micrographs of purified MCP-Tb and MCP-rbc

The purified MCP-Tb (a) and MCP-rbc (b) were negatively stained with 1% uranyl acetate, and examined under a transmission electron microscope at a magnification of 105 000. The arrows show MCP-Tb units associated to form long ribbon-like chains. The diluted samples of MCP-Tb and MCP-rbc are shown in (c) and (d) respectively.

4.8.2 2D-PAGE analysis

In view of the observed differences in relative molecular mass between native MCP-Tb and MCP-rbc (Fig. 3.4), 2D-PAGE was carried out to evaluate possible differences between their subunit composition (Fig. 4.2).

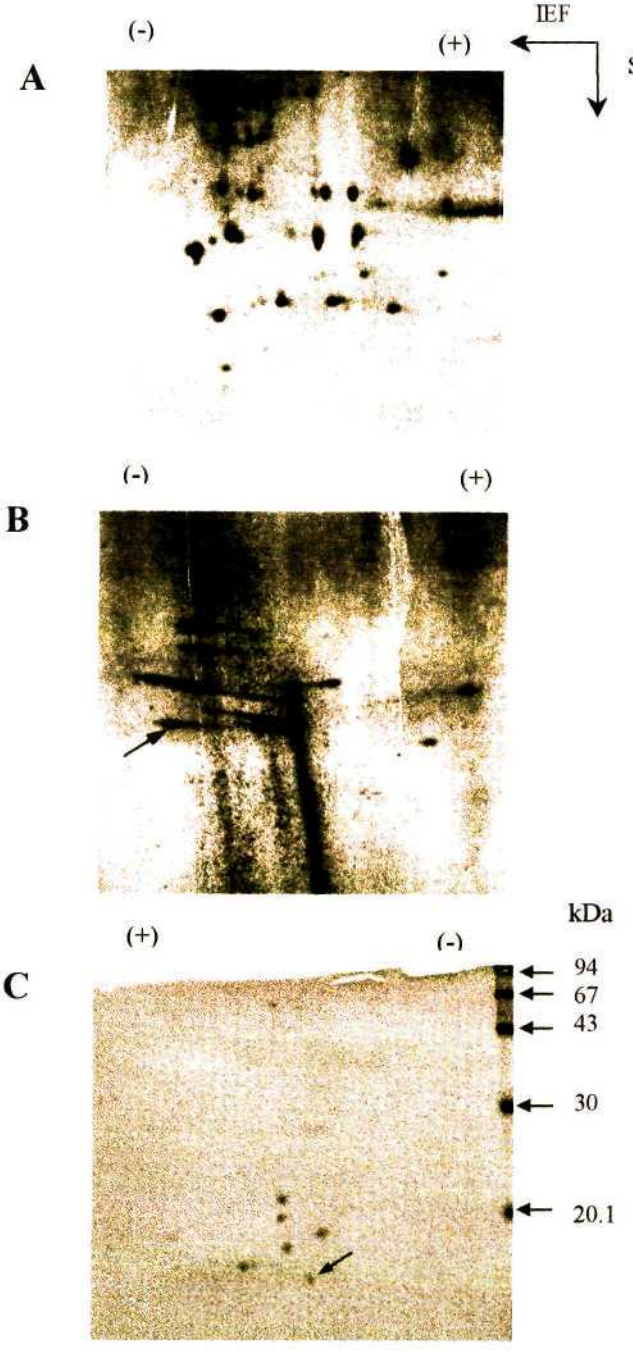

Figure 4.2 Characteristic 2D-PAGE patterns of subunits of MCP-rbc and MCP-Tb

Purified MCP-rbc (A) and MCP-Tb (B) were subjected to 2D-PAGE and silver stained. Some polypeptide components of MCP-Tb (C) do not stained with Coomassie Blue. M_r markers (C) are as shown in the legend to Figure 3.4B. The arrow indicates the polypeptide component blotted for N-terminal microsequencing.

The MCP-rbc appears to have a more complicated pattern of spots on the two-dimensional gel (Fig. 4.2A). There were at least 28 different polypeptide components on the 2D-PAGE of MCP-rbc which were more numerous than those obtained for MCP-Tb (Fig. 4.2B). The relative molecular masses of the MCP-rbc subunits ranged from 21 to 34 and they had a wider range of pI-values than those of MCP-Tb of between 4 to 10.

The two-dimensional gel revealed 12 spots of protein components of MCP-Tb when silver-stained. An arrow shows the spot of protein component sequenced (Fig. 4.2B and C). Some protein components did not stain with Coomassie Blue (4.2C). The relative molecular masses of the protein components range from 17 to 27 kDa and have a wide range of pI values between 4 and 7. Protein components are concentrated towards the basic end of the pH gradient. The pattern of polypeptide components was highly reproducible, although the spots differ in intensity when silver-stained or Coomassie Blue-stained. The 2D-PAGE also facilitated the separation of subunits of MCP-Tb for subsequent N-terminal microsequencing.

4.8.3 N-terminal sequence

The subunit selected for N-terminal sequencing is indicated in Fig. 4.2 (arrow). Selection was based on the observation by Rivett (1993) that this subunit is not N-terminally blocked and could therefore be subjected to N-terminal sequencing directly. The 12 N-terminal amino acid residues of MCP-Tb identified were LXAIXQYAKIXV. Cysteine residues are not normally detected by the PTH-derivative analyser hence appear on the chromatogram as blank 'X'. Residues 2, 5 and 11 in the sequence may be cysteine residues.

A FASTA search for sequence similarity in the SWISS-PROT database found no matches. However, the N-terminal sequence had some (albeit minimal) similarities within β-type sequences of proteasomes from different species. Sequences of the N-terminal regions of different proteasomal β-type subunits share some conserved regions but have variable lengths at the N-terminus (Fig. 4.3). The third (Ala) and the fourth (Ile) residues from the N-terminus are conserved in Human Hs-C5, Human Hs-10 and *Saccharomyces cerevisiae* Doa3 subunits and the N-terminal sequence for the subunit of MCP-Tb from *T. brucei*.

```
Tb-x        Tb 1  -------- --LXAIXQYA KIXV--
Hs-C5       Hs 1 rFSPYVFNG GTILAIAGED FAIVA-
Doa3        Sc 1  -------- --MQAIADSF SVPNR-
Hs-10       Hs 9  gAVMAMKG KNCVAIAADR RFGIQ-
Hs-N3       Hs 9  TSVLGVKF EGGVVIAADM LGSyG-
M1-PrcB     Ml 1  TTIVVLKY PGGVVIAGDR RSTQG-
Ec-Hs1V     Ec 1 mTTIVSVRR NGHVVIAGDG QATLG-
LMP7        Hs 1  TTTLAFKF QHG.VIAAVDSRASAG-
Hs-epsilon  Hs 1  TTTLAFKF RHG.VIVAADSRATAG-
MECL1       Hs 1  TTIAGLVF QDG.VILGADTRATND-
Ta-beta     Ta 1  TTTVGITL KDA.VIMATERRVTME-
```

Figure 4.3 Comparison of N-terminal sequences of a β-type subunit of MCP-Tb with other possibly related proteins.

Different β-type subunits of MCP were aligned with the N-terminal sequence of MCP-Tb designated Tb-x. The second column shows the species *Escherichia coli* (Ec), *Mycobacterium leprae* (Ml), *Trypanosoma brucei* (Tb), *Thermoplasma acidophilum* (Ta), *Saccharomyces cerevisiae* (Sc), and *Homo sapiens* (Hs). Protein sequences indicated are subunits of preoteasomes from the organisms shown. The numbering corresponds to the amino acid residues in the protein. Dashes indicate gaps introduced for better alignment. Single-letter abbreviations for amino acid residues have been used and similar residues are shaded gray. 'X' may be cysteine residues.

4.8.4 Antibody profile

The progress of antibody response to the respective immunogens, was followed by ELISA analyses (Section 2.12) of IgY extracted from egg yolks collected at different time intervals (Fig. 4.4). The increase in antibody titre, which is expressed here as the concentration of antibody at which A_{405}-values are significantly higher than those of equivalent pre-immune controls, is shown for the course of the inoculation period. The two chickens inoculated with the same antigen showed a similar progress of antibody production with minor variations in antibody titre. The profile of primary antibody response has been shown for one chicken for each antigen.

The chickens responded well during the course of the immunization program. There was a quicker antibody response against VSG (Fig. 4.4A) than against MCP-Tb (Fig. 4.4B) but the titre of the latter was higher (large increase between weeks 6 and 9, and decrease from weeks 9 to 12). Immunization of chickens with bovine PA700 showed a slower antibody response (4.4C) compared to MCP-Tb and VSG antigens.

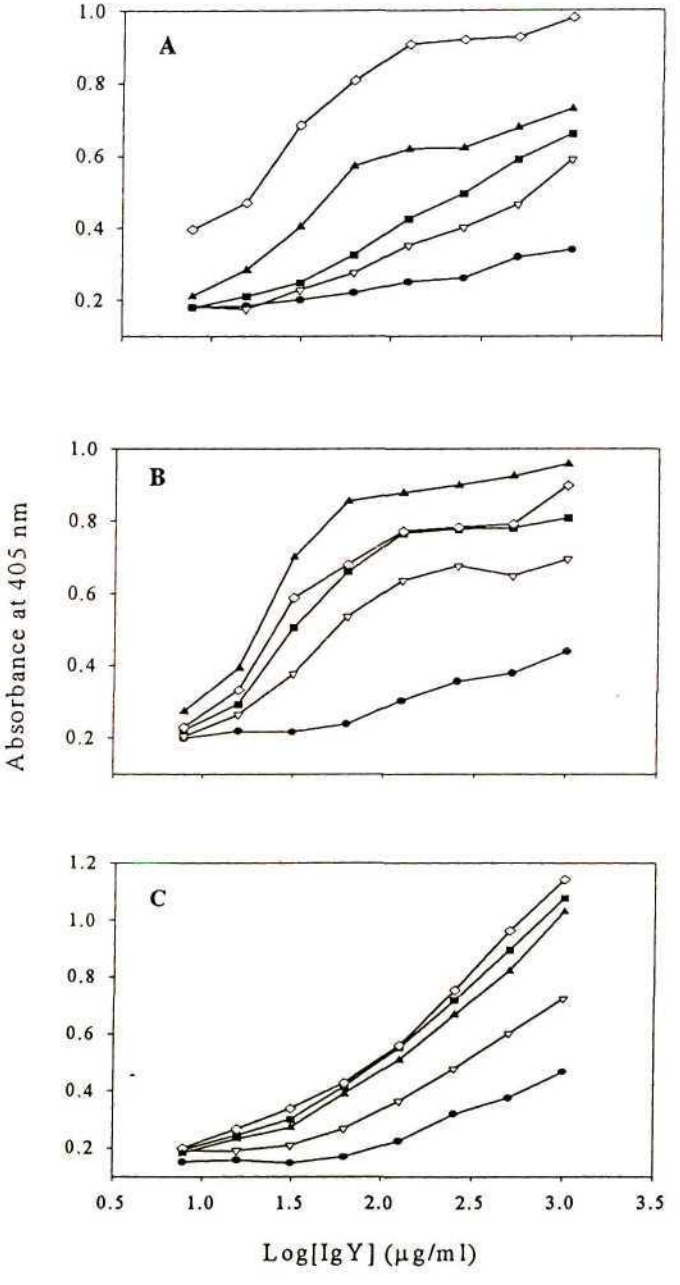

Figure 4.4. Progress of immunization of chickens with *T. brucei* VSG, MCP-Tb and PA700 as determined by ELISA

Parasite proteins (1 µg/ml) were adsorbed to the wells of microtitre plates for 16 h at 4°C and incubated with antibodies directed against VSG (A), MCP-Tb (B) and PA700 (C). Antibodies were collected pre-immunization (●) and after 3 (▽), 6 (■), 9 (◇), and 12 weeks (▲). Binding was visualised by incubation with rabbit anti-chicken HRPO-linked secondary antibodies as described in Section 2.12. Each point is the mean absorbance at 405 nm of duplicate samples.

The antibody production peaked for MCP-Tb in week 12 with a titre of 15 $\mu g.ml^{-1}$ and for VSG with a titre of 12 $\mu g.ml^{-1}$ in week 9 although the antibody had not titrated to completion. The peak antibody production against PA700 was 60 $\mu g.ml^{-1}$ at week 6 that had a similar antibody titre to week 9 (4.4C). There was a decrease in antibody production against PA700 in week 12 of the inoculation programme.

4.8.5 SDS-PAGE and Immunoblot analyses of VSG

Trypanosomes usually overcome the immune capabilities of a host animal by undergoing antigenic variation, a phenomenon that involves sequential replacement of variant surface glycoproteins (VSG). VSGs were purified from *T. brucei* and the purity of VSG assessed by SDS-PAGE. They showed a single band of relative molecular mass of 65 kDa (Fig 4.5A).

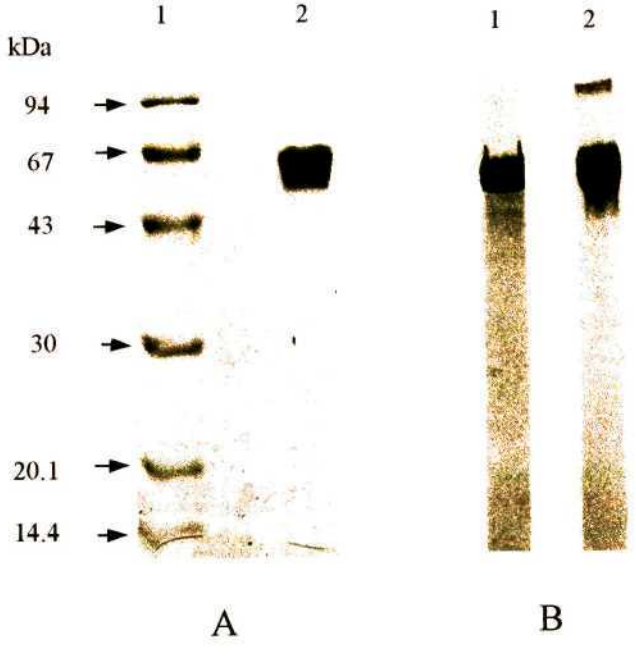

Figure 4.5. Electrophoretic and immunoblot analyses of purified VSG.

Panel A, reducing SDS-PAGE of M_r markers (lane 1), as in Legend to Fig. 3.4 and VSG (lane 2). The gel was silver stained (Section 2.6.3). *Panel B*, purified VSG (lane 1) and crude trypanosome lysate (lane 2) were electroblotted from reducing SDS-PAGE onto nitrocellulose and probed by chicken anti-VSG antibodies. The bound antibodies were detected as described in Section 2.11.2.

Anti-VSG antibodies raised in chickens recognized both purified VSG (Fig. 4.5B, lane 1) and VSG in a crude trypanosome lysate (not shown) separated on SDS-PAGE. This

indicated that the anti-VSG antibodies were specific since they did not cross-react with any protein in the crude lysate. These antibodies were used in immunolocalisation studies reported in Chapter 5.

4.8.6 Western blot of MCP-Tb

Fig 4.6A shows western blotting experiments of the targeting of purified MCP-Tb (lane 1) and MCP-rbc (lane 2) electrophoresed on a non-denaturing PAGE by chicken anti-MCP-Tb IgY. Fig. 4.6B shows a blot of polypeptide components of MCP-Tb and MCP-rbc separated by SDS-PAGE. The antibodies recognized only 27 kDa subunits of MCP-Tb separated on SDS-PAGE and did not recognise any of the subunits of MCP-rbc.

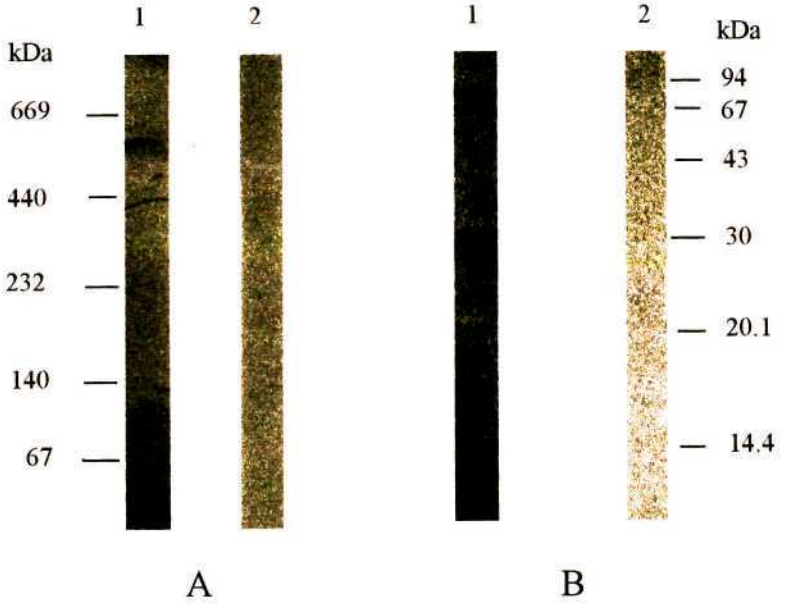

Figure 4.6. Western blot analyses of MCP-Tb and MCP-rbc with chicken anti-MCP-Tb IgY
Purified MCP-Tb (lane 1) and MCP-rbc (lane 2) were electroblotted from non-denaturing PAGE (*Panel A*) and reducing SDS-PAGE (*Panel B*) onto nitrocellulose and probed with chicken anti-MCP-Tb IgY. The bound antibodies were detected in both *Panels A* and *B* as described under Section 2.11.2. The positions of M_r markers are indicated.

4.8.7 Inhibition of MCP-Tb activity by chicken anti-MCP IgY

The effect of anti-MCP-Tb antibodies on the proteolytic activities of purified MCP-Tb and MCP-rbc was tested (Fig 4.7). Although the antibodies had inhibitory effect on the MCP-Tb activity, complete inhibition of the enzyme activity was not observed. Anti-MCP-Tb

antibodies did not inhibit the activity of MCP-rbc. Pre-immune antibodies had no effect on MCP-Tb or MCP-rbc activities.

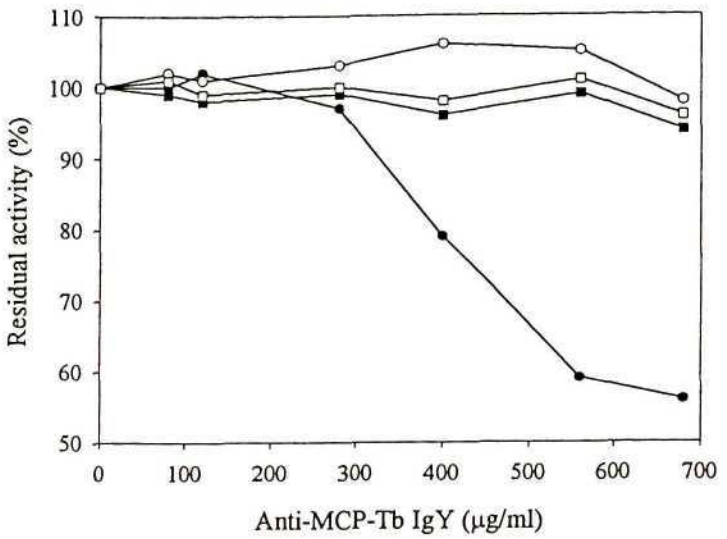

Figure 4.7. Comparative effects of chicken anti-MCP-Tb on MCP-Tb and MCP-rbc hydrolysis of Z-Gly-Gly-Leu-AMC.

MCP-Tb (□ and □) or MCP-rbc (□ and □) was incubated with various amounts of anti-MCP-Tb IgY (□and □) or preimmune IgY (□ and □).

4.9 Discussion

Structural Analysis

MCP-Tb is similar to eukaryotic MCP with respect to size and shape. PAGE analysis of MCP-Tb shows that it has a similar molecular mass (albeit slightly smaller) and complex composition as MCP-rbc. Electron microscopy of negatively stained MCP-Tb preparations suggests a cylindrical structure as in MCP-rbc, rat muscle and liver proteasomes (Baumeister *et al.*, 1988, Djaballah *et al.*, 1993). Particles viewed end-on appears to be ring-shaped and side-on a rectangular shape with four striations, suggesting a characteristic stack of four rings. MCP-Tb, like the MCP-rbc, has a six-fold symmetry. Thus, morphological similarities between MCP-rbc and MCP-Tb suggest that MCP-Tb is equivalent to the 20S proteasome and unlike the high molecular mass tricorn protease (Tamura *et al.*, 1996) or the recently described giant protease (Geier *et al.*, 1999).

A unique feature observed for MCP-Tb is the end-on association of the MCP-Tb units to form long ribbon-like chains. The implication of this end-on interaction between the MCP-Tb units is not certain at present, neither has it been reported in other proteasome preparations. However, it is likely that MCP in trypanosomatids may exists in "intestine-like organelles" rather than singly as the characteristic "meat-grinder" appearance of other proteasome. This end-on association of the MCP-Tb units may have a significant functional meaning. For instance, the direct passage of protein substrates from one MCP-Tb unit to another may result in more efficient processing of the substrate compared to MCP-Tb units acting singly. A scenario can thus be envisaged where the MCP activator PA700, caps the end of MCP-Tb only after every ten or more units rather than individual MCP units. This low frequency of PA700 may explain the failure to hitherto isolate an intact PA700 from the trypanosomes.

Analysis of the subunit composition of MCP-Tb revealed 12 spots. This differs from the observations of Hua *et al.* (1996) that reported 8 different components of a similar *T. brucei* enzyme resolved on a 2D-PAGE. In contrast to MCP-Tb, MCP-rbc showed at least 28 different protein components. These differences may be partly explained by the fact that different polypeptides are not always present in equal amounts and that the number appears to vary depending upon the species (Rivett, 1993). For example, yeast proteasomes have 14 different types of subunits (Heinmeyer *et al.*, 1991), plant proteasomes 12 to 15 (Schliephacke *et al.*, 1991) and other proteasomes have been reported to have 25 distinct polypeptides (Rivett and Sweeney, 1991, Zwickl *et al.*, 1992, Chen and Hochstrasser, 1995). This contrasts with the simple pair of subunits (α, 27 kDa and β, 25 kDa; Dahlmann *et al.*, 1989) that makes up the archaeon *T. acidophilum*, proteasome. A natural question is whether the population of eukaryotic proteasomes is composed of a mixture of biochemically related particles with varied subunit composition or whether the various subunits not related to each other combine in a single complex. It is likely that the differences in polypeptide composition may be due to differences in proteasome complexity. It is also possible that some subunit heterogeneity is not always detected by 2D-PAGE, the standard method of analysis, or that the supposed subpopulations of proteasomes may actually contain additional factors that are not part of the 20S particle proper. Further, the differences in the number of polypeptides appearing on the 2D-gel may arise from proteolysis of some subunits of proteasome during purification, storage or activation of the enzyme (Rivett and Sweeney, 1991, Weitman and

Etlinger, 1992). Phosphorylation (Haass and Kloetzel, 1991) or glycosylation (Schliephacke *et al.*, 1991) may alter the properties of particular subunits.

For the purpose of comparison of the primary sequence of MCP-Tb subunit with the protein sequences in the database, a protein component of MCP-Tb (Tb-x) was selected and its N-terminal sequence determined. The spot selected for N-terminal sequencing from the 2D-PAGE of MCP-Tb was presumed to be a β-type subunit. The β-type subunits have been shown to migrate to the bottom end of the two-dimensional gel (Zwickl *et al.*, 1992, Chen and Hochstrasser, 1995) and are generally not N-terminally blocked (Rivett, 1993). It has also been shown that the β-type subunits possess the proteolytic activity in the proteasome (Seemuller *et al.*, 1995). As predicted, Tb-x was found not to be N-terminally blocked.

In general, sequence similarities and sequence alignments are theoretical sequence analyses that are biologically important. Similarity of a pair of amino acid sequences may provide insight into evolutionary relationships, allow the function and sometimes the structure of the protein to be mapped in relation to well-characterised proteins. Multiple alignment can be used to identify the best conserved sequences or structural motifs. For instance, yeast Doa3 protein has extensive sequence similarity to 20S proteasome β-type subunits and has been implicated in the chymotrypsin-like activity of the yeast proteasome (Chen and Hochstrasser, 1995). Detection of these homology domains in uncharacterized protein sequences may give valuable clues to the biological role of the protein. However, sometimes homology regions do occur in several otherwise unrelated proteins (Asano *et al.*, 1997; Lupas *et al.*, 1997). Nevertheless, such homology regions frequently confer a specific functionality to the proteins. In the present experiment alignments were done and resulted in the listed sequence comparisons (Fig. 4.3). Clearly, the alignment homologies are weak, but since the proteins all have only weak homologies with respect to each other, and since all have proteasome associations, it is not surprising that the protein from MCP-Tb is not overly like any of the others.

A longer continuous sequence of the MCP-Tb subunit would provide a better picture of the sequence similarity when aligned with sequences of other proteasome subunits. Further, it is possible that the lack of similarity at the amino-terminus of the mature β-type subunits is a consequence of post-translational modifications of the subunits. For instance,

mammalian proteasome subunit Lmp7 has been shown to be proteolytically processed from a ~30 kDa precursor to a mature form of ~23 kDa found in 20S particles (Glynne *et al.*, 1993). Similarly, the yeast Doa3 subunit is cleaved post-translationally to produce the mature form detected in purified proteasomes (Chen and Hochstrasser, 1995). In both cases the precursor subunits are amino-terminally processed but the exact position of processing is uncertain. As noted in the alignment results, the sequences have variable lengths attributed to the prosequences cleaved off in mature proteins. The prosegments of the proteasome subunit precursors have been suggested to play a role in preventing premature enzyme activation and may also be important for sub-cellular localization (Früh *et al.*, 1992, Glynne *et al.*, 1993).

Related proteins retain the same basic three-dimensional fold and functionally important residues, long after evolutionary divergence may have led to a randomization of the amino acid sequence at non essential positions (Akiyama *et al.*, 1994, Lowe *et al.*, 1995). Thus, absence of domains homologous to the N-terminal sequence of MCP-Tb (Tb-x) subunit may imply that the sequence is part of a less conserved, non-essential region in the enzyme rather than the sequence of a novel molecule. N-terminal sequencing of further subunits as well as those of internal peptides obtained by limited hydrolysis of subunits will clarify sequence similarities between MCP-Tb and proteasomes from other species.

Immunochemical studies

For the purposes of different experiments in this study, antibodies were raised against MCP-Tb and VSG isolated from *T. brucei* as well as the proteasome activator PA700 isolated from bovine host erythrocytes. MCP-Tb was found to be highly immunogenic in chickens. By week six post-inoculation, a high titre of antibodies was produced. Interestingly, VSG that has a molecular mass of 65 kDa (Cross, 1975) elicited a higher titre of antibodies compared to MCP-Tb that has a native M_r of 590 kDa. This suggests that the immunogenicity of the antigens depends more on the nature of the inoculated substance than on the molecular size of the immunogen. The VSG has a glycolipid component (Englund, 1993) that probably makes it more immunogenic. On the other hand, MCP-Tb consists of an assembly of protein subunits with no evidence of a non-protein component. The bovine proteasome activator PA700 was less immunogenic compared to the *T. brucei* derived immunogens.

Chicken anti-MCP-Tb antibodies reacted avidly with purified MCP-Tb, as well as the enzyme in a crude lysate separated by non-denaturing PAGE (Fig. 4.6), but did not react with MCP-rbc on a blot. Although the parasite MCP-Tb and bovine host MCP-rbc have functional and morphological similarities (Sections 3.5.2 and 4.8.1), the lack of immunological cross-reactivity suggests that the two enzyme complexes have undergone evolutionary divergence to become antigenically distinct. Interestingly, anti-MCP-Tb antibodies did not recognize all subunits of MCP-Tb separated by SDS-PAGE. This was thought to be a reflection of the differential immunogenicity of the different subunits of MCP-Tb. This may be the result of the relative exposure of respective MCP-Tb subunits to the immune system of the chickens. The hidden epitopes are probably those of the β-type subunits in the hydrophobic channels of MCP-Tb. In agreement with our observation, polyclonal antibodies raised against the MCP isolated from other sources (Tanaka *et al.*, 1986, McGuire *et al.*, 1988) do not recognize all the polypeptide components separated by SDS-PAGE. The antibody binding to individual subunits of MCP is relatively specific. Antibodies raised against β-type subunits recognize the β-type subunits on the native MCP and do not cross react with α-type subunits (Rivett and Sweeney, 1991). This has enabled elucidation of topography and the arrangement of α- and β-type subunits of MCP (Dahlmann *et al.*, 1999).

Studies have shown that anti-enzyme antibodies may activate, inhibit or have no effect on the enzyme activity depending on the enzyme under study, the region targeted by the antibody and the substrate used to assess the antibody effect (Dingle *et al.*, 1971, Edwards *et al.*, 1990, Coetzer *et al.*, 1992; Troeberg *et al.*, 1997). The anti-MCP-Tb antibodies produced in the present study had an inhibitory effect on the MCP-Tb but had no effect on MCP-rbc activity. This differential targeting of parasite and host MCPs bodes well for developing a vaccine using MCP-Tb. The pre-immune antibodies had no effect on MCP-Tb activity. The inhibitory effect on MCP-Tb activity is likely to be due to specific binding of polyclonal antibodies to the MCP-Tb. However, it is not clear whether the inhibition is due to occlusion of the substrate binding or active site (an unlikely scenario in view of the buried position within the core of the complex), occlusion of the entrance to the core, or simply a distortion of the complex. The antibody bound to the regulatory or catalytic subunit of MCP-Tb may cause conformational changes leading to alteration in the substrate binding or active site of the enzyme (Djaballah *et al.*, 1993).

A contrasting observation was made by Rivett and Sweeney (1991) who observed no effect of anti-MCP antibodies on proteolytic activities of MCP against small synthetic peptide substrates and even on protein degradation. It is notable that they used rabbit IgG raised against rat liver MCP. Thus, the discrepancy in the two observations could be ascribed to different sources of MCP, differences in species in which the antibodies were raised, or differences in complexities of the trypanosomal and rat liver MCPs (Arnon and Shapira, 1967, Coetzer *et al.*, 1992, Rivett, 1993).

The physiological significance, if any, of the production of enzyme inhibitory antibodies by the host against the parasite enzyme is still unknown. In some studies, proteases such as trypanopain-Tc from *T. congolense* and cruzipain from *T. cruzi* have been recognized as major antigens in cattle and human infections respectively (Authié *et al.*, 1992; de Souza *et al.*, 1990). A positive correlation has been shown between the production of antibodies against Trypanopain-Tc and trypanotolerance in cattle (Authié *et al.*, 1993) suggesting that such antibodies may help protect the infected host. The mode by which the antibodies to the parasite protease may protect the host is still unclear. However, the antibodies may kill the parasite through complement activation or interfere with the physiology of the parasite through inhibition of the parasite enzyme.

The present study has shown that the parasite MCP-Tb and the host MCP-rbc have no discernible structural differences under the electron microscope yet they are immunologically distinct. Furthermore, anti-MCP-Tb antibodies inhibit MCP-Tb activity but do not affect the MCP-rbc activity. The latter was not unexpected since anti-MCP-Tb antibodies were found not to cross react with MCP-rbc in a western blot. The use of specific antibodies, and anti-peptide antibodies in particular, is a common tool for the study of an enzyme's function *in vivo*. Anti-peptide antibodies targeted to a specific primary sequence of an enzyme can often give invaluable information as to the contribution of an enzyme to a particular physiological function. The N-terminal sequence of a subunit of MCP-Tb obtained in this study may be used in the design of a peptide sequence for further investigation of MCP-Tb functions *in vivo*. While immunological differences between MCP-Tb and MCP-rbc bode well for vaccine development, there are limitations with regard to the location of the target molecule in the parasite. A potential vaccine target should be either a secreted molecule or a molecule

exposed on the surface of the parasite. To this end, a study was conducted using the high titre specific antibodies prepared against MCP-b and VSG to localise MCP-Tb and VSG in *T. brucei* sections as discussed in Chapter 5.

Chapter 5

Localisation of MCP-Tb in *Trypanosoma brucei*

5.1 Introduction

The multicatalytic proteinase (MCP) is a non-lysosomal protease complex (for reviews; Coux *et al.*, 1996, Tanaka *et al.*, 1998). In eukaryotic cells, MCP has been localised in the cytoplasmic matrix, in association with the endoplasmic reticulum, and in the nucleus (Akhayat *et al.*, 1987; Tanaka *et al.*, 1989; Peters *et al.*, 1994; Palmer *et al.*, 1996). The heterogeneous localization of MCP is consistent with its proposed multiple functions in intracellular protein turnover and RNA processing (Pouch *et al.*, 1995). However, no report is available with regard to the relative distribution of MCP in trypanosomatids. The purpose of the investigation reported in this chapter was to determine the subcellular localisation of the MCP complex in bloodstream forms of *T. brucei*. The localisation of MCP in these parasites was evaluated by two different techniques. Firstly, parasites were disrupted by a French Pressure Cell and the homogenate obtained subjected to differential centrifugation. The subcellular fractions thus obtained were assayed for MCP-Tb activity. The subcellular fractions were also screened for the presence of putative organelle marker enzymes. The second approach to localising MCP in *T. brucei* involved immunocytochemistry at the electron microscopy level using polyclonal antibodies raised against the purified MCP-Tb.

Immunocytochemistry is an accurate method for subcellular localisation and characterisation of different molecules. This technique has been used successfully to localise intracellular proteins including MCP in rat tissue (Kamakura *et al.*, 1988; Palmer *et al.*, 1996), *Drosophila* (Haass *et al.*, 1989), sea urchin (Akhayat *et al.*, 1987) and newt (Peters *et al.*, 1994). The success of cytochemical experiments depends on the specificity of the antibodies, the resin used for embedding, and the potency of immunogold probes.

The specificity of the polyclonal antibodies raised against MCP-Tb and VSG to their respective antigens was illustrated in Sections 4.13 and 4.11 respectively. The resins used in the present experiments are low temperature (Lowicryl) embedding media chosen with regard to their ability to enable retention of antigenicity and preservation of the

ultrastructure of the specimen (Roth *et al.*, 1981; Hobot, 1989). It is believed that maintenance of low temperature during processing of specimens is a valuable aid to not only the preservation of ultrastructure, but also to the retention of soluble components in their *in vivo* positions and conformations (Roth *et al.*, 1981; Weibull and Christiansson, 1986). Different resins that can be used for tissue embedding at low temperatures have been developed. One such embedding media is the Lowicryl resin.

Lowicryl resins are acrylate-methacrylate mixtures that form a vinyl type of carbon backbone during polymerisation (Carlemalm *et al.*, 1985). Properties that make Lowicryl resins suitable for use at low temperatures include miscibility with different organic solvents, low viscosity, and ability to be polymerised by ultraviolet light. Lowicryl K4M is polar and Lowicryl HM20 is non-polar and these can be used at -35°C and -50°C respectively, while Lowicryl KIIM and Lowicryl HM23 are the respective polar and non-polar ultra-low temperature equivalents (Acetarin *et al.*, 1986). Lowicryl K4M can be kept in a partially hydrated state during dehydration and infiltration, since the resin can be polymerised with up to 5% (m/m) water in the resin block. Low temperature embedding may be achieved by the progressive lowering of temperature (PLT) technique (Kellenberger, 1985). This technique involves stepwise reduction in temperature as the concentration of the dehydrating agent is increased.

Once the specimen sections are cut from the embedding media, enzyme or gold labeling techniques may be employed to facilitate the visualization of intracellular components. Gold labeling systems take a number of forms. An immunogold label entails the coupling of colloidal gold to an antibody. This antibody-gold conjugate may be used directly where the gold particle is bound to a primary antibody, which will bind to the antigen of interest, or the conjugate may be used as a secondary antibody which then binds to a primary antibody bound to the targeted antigen. The latter technique has been used in this study for it has the advantage of an amplifying effect, as many secondary antibodies, and thus many gold particles bind to the primary antibody (Merighi, 1992).

5.2 Cell Fractionation

5.2.1 Reagents

SHADE Buffer (250 mM Sucrose, 50 mM Hepes, 2 mM ATP, 5 mM Na_2EDTA, pH 7.4). Sucrose (8.56 g), Hepes (1.19 g), ATP (0.11 g), Na_2EDTA (0.19 g) were dissolved in 90 ml of dist.H_2O, adjusted to pH 7.4 with NaOH and the volume made up to 100 ml with dist.H_2O.

5.2.2 Procedure

Cells were fractionated by differential centrifugation as previously described (Grab *et al.*, 1987). Trypanosomes (approx.1 x 10^9 cells) were washed in SHADE buffer at 4°C. The parasites were disrupted by a French Pressure cell under chamber pressure of 2500 psi. The homogenate was centrifuged (705 x g, 10 min, 4°C). The resulting pellet designated the crude nuclear fraction (NU) contained unbroken cells, nuclei and cell debris. The resulting supernatant was centrifuged (2 800 x g, 10 min, 4°C) to produce a large granule (LG) fraction. This was followed by a small granule (SG) fraction produced from centrifugation (15 000 x g, 10 min, 4°C) of the post-LG supernatant. The last centrifugation step (123 000 x g, 90 min, 4°C) in a 75 Ti rotor produced the crude microsomal (MI) pellet fraction and the cytosolic (CY) supernatant. All pellets, with exception of MI, were washed with the same buffer and the washings incorporated into the next centrifugation step.

5.3 Determination of enzyme activities

5.3.1 Acid Phosphatase

5.3.1.1 Reagents

Assay buffer [100 mM Na acetate buffer, pH 5.0]. Glacial acetic acid (572 µl) was added to 80 ml of dist.H_2O, adjusted to pH 5.0 with NaOH and made up to 100 ml with dist.H_2O.

Para-nitrophenyl phosphate (50 mM Na_2 *p*NPP). Na_2 *p*NPP (65.8 mg) was dissolved in 5 ml assay buffer.

Stopping solution (10 mM NaOH). NaOH (40 mg) was dissolved in 100 ml of dist.H_2O.

5.3.1.2 Procedure

Acid phosphatase (EC 3.1.3.2) activity was assayed using *p*-nitrophenol phosphate (*p*NPP) as a substrate essentially as described previously (Tosomba *et al.*, 1996). The reaction mixture consisted of assay buffer (25 µl), *p*NPP (5 µl) and 20 µl enzyme solution made up in a 96-well microtitre plate. The reaction was stopped by the addition of 10 mM NaOH (200 µl). The amount of *p*-nitrophenol liberated was measured spectrophotometrically at 405 nm using a BIO-TEK EL 312 microtitre plate reader. One unit of enzyme was defined as the amount liberating 1 µmole of *p*-nitrophenol per minute under the above conditions. An extinction coefficient of 1.465 $mole^{-1}.ml.cm^{-1}$ was used to determine the number of units of the *p*-nitrophenol (Tosomba *et al.*, 1996).

5.3.2 Cysteine protease

5.3.2.1 Reagents

Assay buffer (100 mM Mes, 2 mM Na_2EDTA, 6 mM DTT, pH 6.0). Mes (1.952 g), Na_2EDTA (74.4 mg) and DTT (6.7 mg) were dissolved in 80 ml dist.H2O, adjusted to pH 6.0 with NaOH and made up to 100 ml.

Substrate stock solution (1 mM Z-Phe-Arg-AMC). Z-Phe-Arg-AMC (1.3 mg) was dissolved in DMSO (2 ml). Aliquots (100 µl) were kept at –20°C. The substrate was diluted with assay buffer to a working concentration of 0.1 mM before use.

5.3.2.2 Procedure

Cysteine protease activity was measured with substrate Z-Phe-Arg-AMC as described (Mbawa *et al.*, 1992). Briefly, the incubation mixture consisted of assay buffer (50 µl), Z-Phe-Arg-AMC (25 µl) and enzyme protein solution (10 µl) in a total volume of 100 µl. The reaction proceeded for 15 min at 37°C. The release of fluorophore was monitored as outlined in Section 3.4.1.

5.3.3 Mitochondrial ATPase

5.3.3.1 Reagents

Assay buffer (500 mM Tris-acetate, 100 mM $MgSO_4$, pH 7.4). Tris (6.055 g) and $MgSO.7H_2O$ (2.464 g) were dissolved in 80 ml of dist.H_2O, adjusted to pH 7.4 with acetic acid and made up to 100 ml with dist.H_2O.

ATP solution (200 mM Na_2ATP). $Na_2ATP.3H_2O$ (0.121 g) was dissolved in 1 ml of assay buffer.

Phosphoenolpyruvate solution (50 mM Na_3PEP). Trisodium phosphoenolpyruvate (58.5 mg) was dissolved in 5 ml of assay buffer.

Oligomycin solution (1mg/ml oligomycin). Oligomycin (5 mg) was dissolved in 5 ml of assay buffer.

50% (v/v) TCA. TCA (10 g) was dissolved in 20 ml of dist.H2O.

Ascorbic-molybdate solution [2% (m/v) $C_8H_8O_8$, 0.42% m/v $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.5 M H_2SO_4]. Ammonium molybdate (0.42 g) and conc. H_2SO_4 (2.86 ml) were dissolved in 80 ml dist. H_2O, transferred to a 100 ml volumetric flask and the volume adjusted to 100 ml with dist. H_2O. This solution was stable at room temperature. The required ascorbic-molybdate solution was made 2% ascorbic acid (reducing agent) just before use (Ames, 1966).

5.3.3.2 Procedures

Total ATPase (EC 3.6.1.3) activity was measured by ATP hydrolysis employing an ATP-generating (phosphoenolpyruvate-pyruvate kinase) assay system (Pullman and Penefsky, 1963). Briefly, the assay mixture consisted of assay buffer (100 µl), ATP (5 µl), $MgSO_4$ (10 µl), phosphoenolpyruvate (100 µl), pyruvate kinase (40 µl) and 20 µl enzyme protein solution, with or without oligomycin (10 µl). Incubations were carried out at 37°C with gentle shaking (50 oscillations/min) for 30 min. The reaction was stopped by addition of 50% (v/v) TCA (100 µl). Blanks were prepared in separate microfuge tubes by stopping the reaction at time zero with 50% (v/v) TCA (100 µl). After centrifugation (15 000 x g;

25°C; 5 min) of the precipitated proteins, an aliquot of the supernatant solution (200 µl) was removed for determination of inorganic phosphate liberated.

The inorganic phosphate was determined by the method of Ames (1966). The clear supernatant (200 µl) was mixed with ascorbic-molybdate solution (800 µl) and incubated for 20 min at 45°C. The inorganic phosphate was measured by monitoring absorbance at 820 nm using a Pharmacia LKB Ultrospec III Spectrophotometer. A molar extinction coefficient of 6.05 $\mu mole^{-1}.ml.cm^{-1}$ was used to determine the number of µmoles of the inorganic phosphates released. The 'mitochondrial' ATPase activity was estimated as the difference between total ATPase activity and the activity resistant to 10 µg oligomycin per ml (Opperdoes *et al.* 1977b).

5.3.4 Glycerol-3-phosphate dehydrogenase

5.3.4.1 Reagents

Assay buffer (158 mM triethanolamine, pH 7.8). Triethanolamine (1.48 g) was dissolved in 30 ml of dist. H_2O, adjusted to pH 7.8 with HCl and made up to 50 ml with dist.H_2O.

10 % Triton X-100. See Section 2.8.1.1

50% (v/v) TCA. See Section 5.3.3.1

Dihydroxyacetone phosphate solution (10 mM DHAP). Dihydroxyacetone phosphate (85 mg) was dissolved in 5 ml of assay buffer.

NADH solution (10 mM NADH). NADH (5 mg) was dissolved in 630 µl of dist.H_2O.

5.3.4.2 Procedure

Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity was assayed spectrophotometrically by following the oxidation of NADH as described (Rovis and Baekkeskov, 1980). The reaction mixture (0.9 ml) consisted of assay buffer (450 µl), NADH (18 µl), dihydroxyacetone phosphate (198 µl), 10 (v/v) Triton X-100 (9 µl) and enzyme protein solution (20 µl). The reaction mixture was incubated for 15 min at 25°C. The reaction was stopped by addition of 50% TCA (100 µl). The mixture was centrifuged

(15 000 x g, 25°C, 2 min) in a microfuge to remove any precipitate. The amount of NADH oxidized was estimated at 340 nm using a Pharmacia LKB Ultrospec III Spectrophotometer. A molar extinction coefficient of 1.109 $mmol^{-1}.ml.cm^{-1}$ was used to determine the number of µmoles of NADH oxidized.

5.3.5 Malate dehydrogenase

5.3.5.1 Reagents

Assay buffer (200 mM Tris, 2 mM Na_2EDTA, pH 7.5). Tris (2.42 g) and Na_2EDTA (74.4 mg) were dissolved in 80 ml dist.H_2O, adjusted to pH 7.5 with HCl and made up to 100 ml with dist.H_2O.

L-malate solution (5 mM DL-Na_2-malate). Malic acid (4.5 mg) was dissolved in 5 ml of dist.H_2O.

Oxidized NAD solution (1 mM NAD^+). NAD^+ (1 mg) was dissolved in 1.5 ml of dist.H_2O.

5.3.5.2 Procedure

Malate dehydrogenase (EC. 1.1.1.37) activity was measured spectrophotometrically by following the reduction of NAD^+ as described (Opperdoes *et al.*, 1977b). The reaction mixture (0.9 ml) consisted of NAD^+(90 µl), L-malate (90 µl), Assay buffer (450 µl) and 10 µl of enzyme protein solution. The reaction mixture was incubated for 15 min at 25°C and the reaction was stopped by addition of 50% (m/v) TCA. (100 µl). The mixture was centrifuged (15 000 x g, 25°C, 2 min). The amount of NADH formed was estimated at 340 nm using a Pharmacia LKB Ultospec III Spectrophotometer. A molar extinction coefficient of 1.109 $mmole^{-1}.ml.cm^{-1}$ was used to determine the number of µmoles NAD^+ reduced.

5.3.6 MCP-Tb

Multicatalytic protease complex (EC 3.4.99.46) activity was measured using Z-Gly-Gly-Leu-AMC as a substrate under conditions described in Section 3.4.1.

5.4 Immunocytochemistry

5.4.1 Lowicryl K4M-embedding of trypanosomes

During this investigation, use was made of initial embedding in gelatin of the fixed, isolated trypanosomes. Initial embedding in gelatin capsules, as opposed to exclusive use of flat embedding in Lowicryl, circumvents a special problem, notably the inhibition of polymerization by atmospheric oxygen due to the large resin surface in contact with air. To further overcome this problem, a relatively airtight chamber was used with N_2 entry and exit ports, so as to create an anaerobic environment within the chamber itself. The N_2 gas is denser than air and when released into the chamber, settles at the bottom expelling air from the chamber.

5.4.1.1 Reagents

Phosphate buffered saline (PBS) (0.01M NaH_2PO_4 0.15 M NaCl, 2.5 mM KCl, pH 7.3). NaH_2PO_4 (1.38 g), NaCl (8.76 g) and KCl (0.186 g) were dissolved in about 800 ml of dist.H_2O, adjusted to pH 7.3 with NaOH and made up to 1 litre.

Hepes stock (400 mM N-2-hydroxyethyl-piperazine N'-2-ethane sulfonic acid, 500 mM sucrose, pH 7.3). Hepes (10.92 g) and sucrose (17.12 g) were dissolved in 90 ml of dist.H_2O, adjusted to pH 7.3 with NaOH and made up to 100 ml. The solution was aliquoted and stored frozen.

10% (v/v) gelatin-PBS. Gelatin (1 g) was dissolved by gentle heating in 10 ml of PBS, pH 7.3

Fixative stock [4% (v/v) paraformaldehyde, 0.1 (v/v) glutaraldehyde in 400 mM Hepes, pH 7.3]. Paraformaldehyde (2.5 ml of 16% stock) and glutaraldehyde (40 µl of 25% stock) were added to about 8 ml of 400 mM Hepes, the pH adjusted to 7.3 if necessary and the solution made up to 10 ml with 400 mM Hepes.

30% (v/v) Ethanol. Ethanol (3 ml, AR) was made up to 10 ml with dist.H_2O.

50% (v/v) Ethanol. Ethanol (5 ml, AR) was made up to 10 ml with dist.H_2O.

75% (v/v) Ethanol. Ethanol (7.5 ml, AR) was made up to 10 ml with dist.H_2O.

95% (v/v) Ethanol. Ethanol (9.5 ml, AR) was made up to 10 ml with dist.H_2O.

Lowicryl K4M resin. Lowicryl K4M resin (Electron Microscopy Sciences, Fort Washington, PA) was formulated essentially as described by the manufacturers. Cross-linker A (2.7 g) and monomer B (17.3 g) were pipetted into an amber coloured glass container on a balance in a fume-hood. Initiator C (0.1 g of solid) was weighed out separately, added to the mixture and dissolved by bubbling N_2 through the resin, which also serves to expel O_2. Care was taken to avoid skin contact or inhaling resin fumes as the resin is thought to cause dermatitis. Each batch of resin could be stored for at least 3 months in the dark at room temperature.

5.4.1.2 Procedure

Bloodstream forms of *T. brucei*, isolated from rat blood (Section 2.3.2) and maintained on ice, were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in 200 mM Hepes buffer (pH 7.3) containing 250 mM sucrose (by addition of equal volume of cells in suspension to stock fixative solution). After fixation for 60 min at room temperature, the fixed cells were pelleted by centrifugation in a microfuge (15 000 x g, 25°C, 3 sec) and fixative drawn off to avoid cross-linking of the gelatin before reaching the cells. The cells were washed (3 x 20 min) with 200 mM Hepes containing 50 mM glycine to quench the free aldehyde groups. The pellet and 10% gelatin-PBS were separately heated to 37°C. All excess liquid in the tubes was removed and 50 µl of warm gelatin added. The pellet was centrifuged again after incubation for 60 min at 37°C. The microfuge tubes were placed on ice to allow gelatin to set and cut into small sizes (approx. 1 mm^3). Cells were subjected to progressive lowering of temperature (PLT) dehydration: 30% ethanol (0°C, ice bath at -4°C, 30 min), 50% ethanol (-20°C, 3 parts crushed ice + 1 part NaCl placed at -10°C, 30 min), 75% ethanol (-35°C, 1.2 parts ice + 2 parts $CaCl.6H_2O$, 30 min), 95% ethanol (-35°C, 30 min), 100% ethanol (-35°C, 30 min, repeated once). Petri dishes were agitated at least twice during each 30 min incubation step. Cells were infiltrated with a 1:1 mixture of resin and 100% ethanol (-35°C, 30 min, 3 ml). This was followed by a 2:1 mixture of resin and 100% ethanol (-35°C, 1 h, 3 ml), pure resin (-35°C, 1 h, 3 ml) and again pure resin (-35°C, overnight, 3 ml). The following day the Petri dishes were placed in heat sinks, resin replaced and the unit flooded with N_2. Resin was allowed to

polymerize in a N_2 atmosphere at –35°C in indirect ultraviolet light (360 nm, 24 h) followed by direct ultraviolet radiation (48 h). Petri dishes were placed on a sunny windowsill for at least 4 days until the resin had properly hardened.

5.4.2 Sectioning of Lowicryl K4M blocks

Lowicryl K4M is a hydrophilic resin, therefore care was taken not to wet the block surface during sectioning. This was accomplished by sectioning with the level of fluid in the knife-trough slightly below the surface, while still maintaining a wet knife-edge. Nickel grids were used (although gold grids could also be used) for labeling, as copper tends to oxidize in the labeling solutions. The stability of sections on the grid during labeling and in the electron microscope was improved by coating grids with formvar.

5.4.2.1 Reagents

0.25% (m/v) Formvar in chloroform. Formvar (0.25 g) was dissolved in chloroform (100 ml).

5.4.2.2 Procedure

A glass dish of about 20 cm diameter was completely filled with dist.H_2O. A clean microscope slide was dipped into the formvar solution, the excess allowed to drain and the remaining film dried. The dried film was loosened around the edges of the slide with a razor blade, and floated onto the surface of the water. Nickel grids were placed, shiny side up, on the floating film. Using a piece of wire mesh as a support, the film was recovered by a scooping motion through the water, leaving the film covering the mesh. Individual formvar-coated grids could then be torn away from the formvar film when required.

Small blocks were cut with a hacksaw from a petri dish containing Lowicryl K4M embedded *T. brucei* cells. The block was mounted onto a plastic stub and the block trimmed on a microtome to a pyramidal face. The ultrathin sections (approx. 90 nm thick) were cut with a glass knife and collected onto formvar coated nickel grids. Grids were dried and could be stored indefinitely before labeling.

5.4.3 Immunolabeling of Lowicryl-embedded sections

Immunolabeling experiments described here made use of the protein A-gold labeling system. Protein A has high affinity for IgGs. Protein A binds to the F_C portion of the

molecule (Forsgren and Sjöquist, 1966), specifically to the C_{H2} and C_{H3} domain (Deisenhofer, 1981) and acts in a pseudo-immune (a non-antigenic type) fashion that does not interfere with the binding of immunoglobulin to its antigen. Protein A interacts strongly with most IgG classes of several mammalian species such as human, rabbit, guinea pig, and dog, and less strongly with IgG classes from goat, sheep and rat (Forsgren and Sjöquist, 1966) and not at all to chicken (Langone, 1982). Where primary antibodies from weaker reacting species are used, a 'linker' secondary antibody from a strong reacting species may be used followed by the protein A-gold probe. Indeed, immunogold probes, where the secondary antibody is adsorbed onto the gold surface instead of protein A, would also be a viable alternative. This offers a further advantage of signal amplification, because more than one immunogold probe may bind to a single tissue-bound primary antibody (Merighi, 1992). Protein A binds immunoglobulins in a one-to-one ratio (Slot *et al.*, 1989), an important factor when quantitative immunocytochemical labeling studies are being undertaken (Griffiths and Hoppeler, 1986).

Labeling of ultrathin sections with protein A-gold, in general follows standard protocols (Merighi, 1992) with the manipulation of variables such as choice of blocking agent, length of incubation times, and length and stringency of washing. The protocol used was essentially as outlined by Roth (1986) except that BSA was used as a blocking agent and grids were washed in drops rather than by spraying.

5.4.3.1 Reagents

2% (m/v) Gelatin-PBS. Gelatin (200 mg) was dissolved in 10 ml PBS (Section 5.4.11).

1% (m/v) BSA-PBS. BSA (globulin-free, 100 mg) was dissolved in 10 ml PBS.

1% (m/v) Glutaraldehyde in PBS. Glutaraldehyde (40 µl of 25% stock) was diluted in 960 µl PBS.

0.02M Glycine in PBS. Glycine (0.015 g) was dissolved in 10 ml of PBS.

Antisera. Chicken anti-MCP IgY, chicken anti-VSG IgY, and rabbit anti-chicken IgY (linker antibody) were diluted in 1% BSA-PBS. The optimal dilution was determined empirically for each of the antibodies. Chicken anti-VSG IgY and anti-MCP IgY were

used at 10 µg/ml and 20 µg/ml respectively. Rabbit anti-chicken IgY linker antibody was used at a 1/200 dilution.

Gold labels. Protein A-gold probes (used at 1/425 and 1/200 dilutions for 5 nm and 10 nm gold probes respectively) were optimized and provided by Dr. Edith Elliot, Department of Biochemistry, University of Natal.

5.4.3.2 Procedure

Immunolabeling was performed by incubation of the grids containing the ultrathin sections prepared as described in Section 5.4.2. Grids were floated, section-side down, on droplets of reagents placed on Parafilm spread onto a bench. During the reaction, droplets were covered with a large glass petri dish to protect them from dust and spillage. The grids were transferred from one reagent droplet to another with a nickel wire loop (0.2 mm diameter, forming a 1 mm loop). The same loop was used for each reagent as the loop carries a small volume of liquid over, which introduces a dilution factor.

Immunogold labeling was performed at 25°C using the following incubation steps:

1) Blocking in 1% (w/v) BSA in PBS (20 µl, 5 min), followed by a 2% (w/v) gelatin droplet (20 µl, 5 min).
2) Labeling in appropriate dilution of primary antibody (10 µl, 2 h).
3) Washing in PBS (20 µl, 5x2 min).
4) Labeling in appropriate dilution of secondary (linker) antibody (10 µl, 1 h)
5) Washing in PBS (20 µl, 5x2 min).
6) Labeling in protein A-gold (5 nm or 10 nm), diluted in BSA-PBS (10 µl, 1 h).
7) Washing in PBS (100 µl, 5x2 min).
8) Fixing in Glutaraldehyde in PBS (10 µl, 5 min).
9) Washing in PBS (100 µl, 2x2 min) followed by dist.H_2O (100 µl, 4x2 min).
10) The grids were gently blotted and allowed to dry.

As a control, all steps were repeated with immune antibody substituted with a non-immune preparation, at the lowest dilution of immune antibody used.

5.4.4 Counterstaining of Lowicryl K4M sections

Lowicryl K4M embedded sections are counterstained in a manner similar to those for traditional electron microscopy resins. Sections may be stained with either aqueous or alcoholic solutions of uranyl acetate and lead citrate (Hobot, 1989). A combination of lead acetate (Millonig, 1961) and uranyl acetate provide superior quality of contrast and fine granular staining (Roth *et al.*, 1990). Trypanosome staining with uranyl acetate and lead citrate provided sufficient contrast. Since Lowicryl K4M is hydrophilic, the sections were incubated on drops of stains for short periods of time. Prolonged staining was found to cause contamination and distortion of the sections.

5.4.4.1 Reagents

2% (m/v) Uranyl acetate. Uranyl acetate (1 g) was dissolved in dist.H_2O (50 ml), and the addition of 95% ethanol (1 ml) to assist solubility. The uranyl acetate solution was stored in the dark at 4°C.

1M NaOH. NaOH (0.4 g) was dissolved in dist.H_2O (10 ml).

Lead citrate. Lead citrate (1.33 g) and trisodium citrate.$2H_2O$ (1.76 g) were added to freshly boiled and cooled dist.H_2O and shaken intermittently for 30 min. NaOH (8 ml) was added to clear the milky solution. The solution was adjusted to 50 ml with freshly boiled and cooled dist.H_2O and stored in the dark at 4°C in an airtight container.

5.4.4.2 Procedure

Dried grids were inverted onto uranyl acetate (4 min, in the dark), washed with dist.H_2O by gentle pipetting (500 µl) and inverted onto lead citrate (30 sec) in a closed glass petri dish containing NaOH pellets to remove CO_2. The grids were again washed with gentle pipetting (500 µl), blotted, allowed to dry and viewed in JEOL 100CX Transmission Electron Microscope at 80 kV.

5.5 Results

5.5.1 Subcellular Fractionation

As a first approach to the subcellular localisation of MCP-Tb in bloodstream form of *T. brucei*, a homogenate was fractionated by differential centrifugation into a nuclear fraction, a large-granule fraction, small-granule fraction, microsomal and soluble cytosolic

fractions. Each of these subcellular fractions was enriched in a particular organelle. The identity of these organelles was determined by assaying for the activity of marker enzymes described in the literature (Opperdoes *et al.*, 1977a; Grab *et al.*, 1987; Mbawa *et al.*, 1991b). The specific activity of MCP-Tb was also determined in each of the five fractions. This allowed the determination of the distribution of MCP-Tb in the various subcellular fractions (Table 5.1).

The highest relative specific activity of acid phosphatase was found in the MI fraction (Table 5.2). This is in agreement with the reported acid phosphatase enrichment in the microsomal fraction of *T. brucei* and *T. congolense* (Steiger *et al.*, 1980; Grab *et al.*, 1987; Tosomba *et al.*, 1996). This enzyme, however, has additional significant activities in the LG, SG and CY fractions, probably reflecting its heterogeneous localisation in the flagellar pocket, lysosomes and in the Golgi apparatus of trypanosomes (Langreth and Balber, 1975; Grab *et al.*, 1987; Tosomba *et al.*, 1996).

Table 5.1 Distribution of MCP-Tb in different subcellular fractions from *T. brucei*
Fractions F_1-F_5 in the order of their isolation are; NU, nuclear; LG, large-granule; SG, small-granule; MI, microsomal; and CY, cytosolic fractions. Distribution is expressed as percentages of the ratios of activity in the fraction over the sum of activities of the five fractions obtained. Values presented are from a representative experiment illustrating how enzyme distribution was determined.

Fraction	Volume *(ml)*	Protein *(mg/ml)*	Total Protein *(mg)*	Activity *(nmol/min/ml)* x 10^{-3}	Total Activity *(nmol/min)* x 10^{-3}	Specific Activity *(nmol/min/mg)* x 10^{-2}	Distribution *(%)*
F_1 (NU)	6.5	4.57	29.71	122.603	796.9	2.68	21.6
F_2 (LG)	1.0	0.95	0.95	9.253	9.25	0.97	0.3
F_3 (SG)	0.7	0.79	0.55	9.996	7.0	1.27	0.2
F_4 (MI)	1.67	2.84	4.74	72.096	120.4	2.54	3.3
F_5 (CY)	36.0	1.18	42.48	76.582	2756.95	6.49	74.7

Parasite plasma membrane including the flagellar pocket, sediments in the NU fraction, whereas lysosomes are enriched in the SG fraction (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, .1991b). The high relative specific activity of acid phosphatase in the MI fraction may thus be attributed to enrichment of Golgi apparatus in the MI fraction. Acid phosphatase was thus adopted as an unequivocal marker enzyme for the Golgi apparatus in the MI fraction.

The commonly used marker enzymes for mitochondrial activity are largely repressed in bloodstream form of *T. brucei* (Vickerman, 1965; Opperdoes *et al.*, 1977a). However, mitochondrial ATPase is active both in bloodstream trypomastigotes and insect forms (Opperdoes *et al.*, 1977b). The mitochondrial ATPase is stimulated by uncouplers such as carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone or 2,4-dinitrophenylhydrazone (Lomo *et al.*, 1996). This activity is also stimulated by Mg^{2+} ions (Opperdoes *et al.*, 1977b). The mitochondrial ATPase is specifically inhibited by oligomycin (Pullman and Penefsky, 1963). Thus, the oligomycin-sensitive portion of the Mg^{2+}-stimulated ATPase activity is referred to as the mitochondrial ATPase in the enzyme distribution studies. The mitochondrial ATPase activity was optimal in the LG fraction, comparable with enrichment of mitochondria in the LG fractions (Opperdoes *et al.*, 1977a).

Table 5.2. Distribution of marker enzymes from *T. brucei* in fractions obtained by differential centrifugation

The experiments were performed as described in Section 5.2.2. Fractions F_1-F_5 are as outlined in the Legend to Table 5.1. Distribution of AcP, acid phosphatase; ATPase, mitochondrial ATPase; CysP, cysteine protease; GPDH, glycerol-3-phosphate dehydrogenase, MCP-Tb, multicatalytic protease from *T. brucei*; and MDH, malate dehydrogenase are expressed as the percentage of specific activity in the fraction over the sum of the specific activities of the five fractions. Total protein in each fraction was determined as described in Section 2.4. Values refer to the means ± standard deviations of three determinations.

Fraction	Distribution (%)						
	Protein	AcP	ATPase	CysP	GPDH	MCP-Tb	MDH
F_1 (NU)	37.9 ± 6.7	16.6 ± 2.7	22.3 ± 4.9	19.2 ± 4.2	14.3 ± 3.5	23.1 ± 5.2	9.7±2.9
F_2 (LG)	3.3 ± 1.2	13.5 ± 3.1	31.8 ± 5.7	25.7 ± 3.5	15.4 ± 7.3	0.3 ± 0.1	4.9 ± 1.4
F_3 (SG)	3.2 ± 2.1	12.4 ± 1.7	18.5 ± 5.3	32.5 ± 4.7	32.6 ± 4.3	0.2 ± 0.1	4.6 ± 1.6
F_4 (MI)	8.3 ± 2.1	34.2 ± 5.5	11.8 ± 6.5	18.4 ± 2.8	20.1 ± 4.4	3.4 ± 0.5	5.4 ± 1.3
F_5 (CY)	54.7 ± 8.0	23.3 ± 8.8	15.6 ± 3.2	17.5 ± 5.0	17.6 ± 3.6	73.0 ± 5.7	75.4 ± 9.8

The distribution profile of cysteine protease activity was as described previously (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, 1991b), showing the highest relative specific activities in the SG fraction. It has been shown by electron microscopy that lysosomes are enriched mainly in SG and partly in the LG fractions of trypanosomes (Grab *et al.*, 1987). The localisation of cysteine proteases in the lysosomes (Lonsdale-Eccles and

Grab, 1987; Mbawa *et al.*, 1991b), therefore, shows that cysteine protease activity is an appropriate marker for lysosomes in trypanosomes.

Glycerol-3-phosphate dehydrogenase (GPDH), an enzyme marker for glycosomes (Opperdoes *et al.*, 1977a; Rovis and Baekkeskov, 1980), presented its maximal specific activity in the SG and MI fractions. This distribution profile is consistent with electron microscopy confirmation that glycosomes are found in the SG and MI fractions (Grab *et al.*, 1987). Low GPDH activity in the soluble supernatant fraction confirms that GPDH is an appropriate marker for intact glycosomes

The distribution profile of MCP-Tb activity shows that a major proportion of specific activity is in the soluble CY fraction and partly in the NU and MI fractions. The LG and SG fractions appear to be devoid of MCP-Tb activity. The low activities of MCP-Tb detected in LG and SG fractions are likely to be from contaminating MCP-Tb.

Malate dehydrogenase (MDH) is typically considered to be a mitochondrial marker enzyme in mammalian cells. In trypanosomes, however, MDH has been shown to be a mitochondrial as well as a microbody enzyme (Opperdoes *et al.*, 1977b). In the present experiment, MDH showed maximal relative specific activities in the particulate NU and soluble CY fractions. The pattern of activity observed in MDH does not conform to the established particulate localisation of the enzyme in *T. brucei* (Opperdoes *et al.*, 1977b). Thus the enzyme could not be used as an organelle marker for mitochondria or glycosomes in these studies.

5.5.2 Immunocytochemistry

A polyclonal antibody was raised in chickens against the purified MCP-Tb and its titre determined by ELISA (Section 4.10). To test for specificity, the antibody was used to probe a western blot of crude trypanosome lysate and purified MCP-Tb (Section 4.13). The antibody was used to label sections of bloodstream form *T. brucei* embedded in Lowicryl K4M resin. Since protein A does not interact with primary antibodies raised in chickens (Forsgren and Sjöquist, 1966; Langone, 1982), a secondary 'linker' secondary antibody, rabbit anti-chicken IgY, was used. Antigen-antibody complexes were visualised using 5 nm protein A gold probes. The antibody strongly labeled the nucleus (Fig 5.1a)

and comparatively low labeling was present in the cytoplasm (Fig. 5.1b). Figure 5.1c shows labeling adjacent to a microbody.

Localisation of VSG in the *T. brucei* section was performed in a three-step immunocytochemical procedure, which had been found to be more sensitive than a two-step procedure. Optimal labeling in the electron microscopic experiments was found when the purified chicken anti-VSG IgY antibodies were used at the dilutions used in the western blot analysis (Section 4.11). After applying chicken anti-VSG IgY, followed by rabbit anti-chicken IgY and protein A gold complex (particle size 10 nm) to the trypanosome section, labeling by gold particles was seen on the flagellar pocket surface and on the surface of the parasite (Fig. 5.2a). The labeling on the flagellar pocket was consistent in most sections observed. Gold labeling was also observed in lysosome-like organelles in the parasite as well as on the surface of the flagella within the flagellar pocket (Fig. 5.2b).

There was good preservation of the ultrastructure of the parasite section embedded in Lowicryl K4M resin (Fig 5.3a). Control incubations with pre-immune IgY instead of the purified primary antibodies (Fig. 5.3b), followed by secondary antibodies and protein A gold, provided evidence of the specificity of labeling. As a control for methodology, glucagon hormone was localised in sections of the pancreas using specific rabbit anti-glucagon antibodies in parallel experiments to those performed with anti-MCP-Tb and anti-VSG antibodies. Labeling was restricted to the β-cells in the pancreas (Fig. 5.3c).

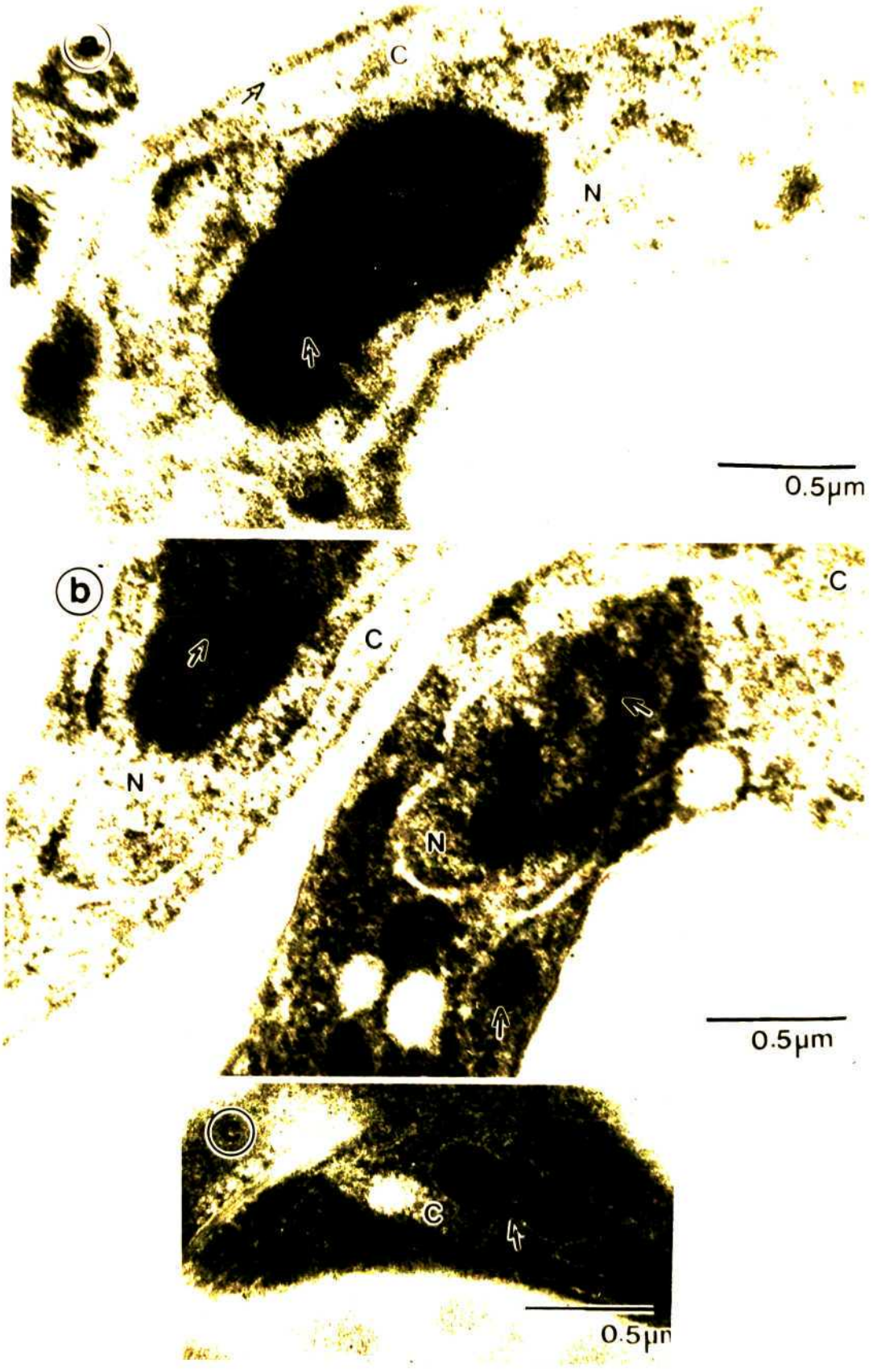
C
N
0.5µm
b
C
C
N
N
0.5µm
C
0.5µm

Figure 5.1. Immunocytochemical localisation of MCP-Tb in bloodstream forms of *T. brucei*.

The Lowicryl K4M embedded sections were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde, 200 mM Hepes, pH 7.3 and labeled with primary (chicken anti-MCP-Tb IgY) antibody, secondary (rabbit anti-chicken IgY) antibody and protein A-colloidal gold (5 nm particle size). (a)– *Arrows* shows gold labeling in the nucleus, N. (b)–Labeling of cytoplasm, C. (c)–Labeling of cytoplasm (*arrow*) around a microbody.

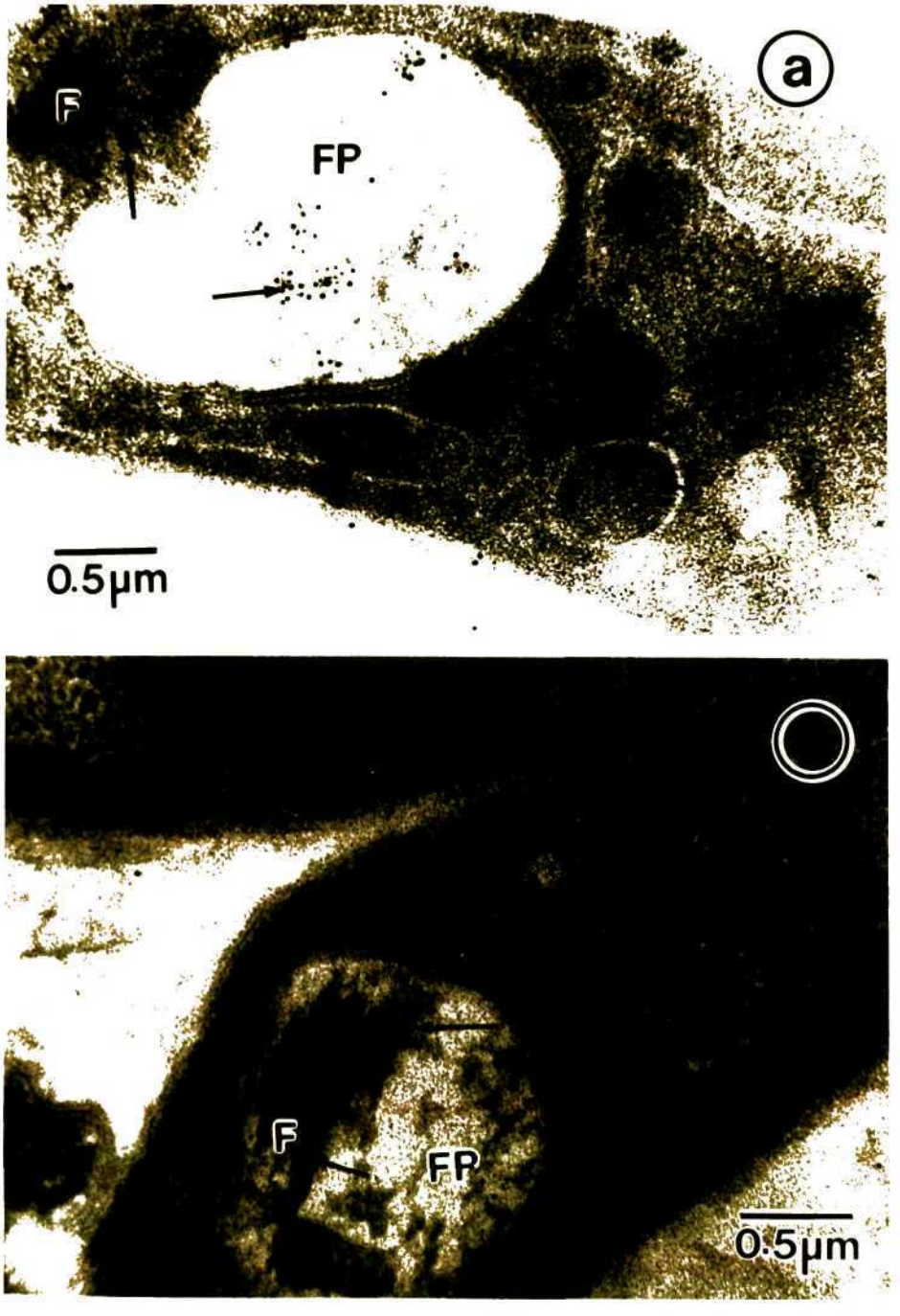
a
F
FP
0.5μm
F
FP
0.5μm

Figure 5.2. Immunocytochemical localisation of VSG in Lowicryl K4M embedded *T. brucei*.
The sections were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde, 200 mM Hepes, pH 7.3 and labeled with primary (chicken anti-VSG) antibody, followed by secondary (rabbit anti-chicken IgY) antibody and protein A gold (10 nm particle size). (a)– Arrows show labeling in the flagellar pocket, FP, and some labeling on the surface of the parasite. (b)– labeling on the flagellum, F and inside a lysosome-like organelle *(arrowed)*

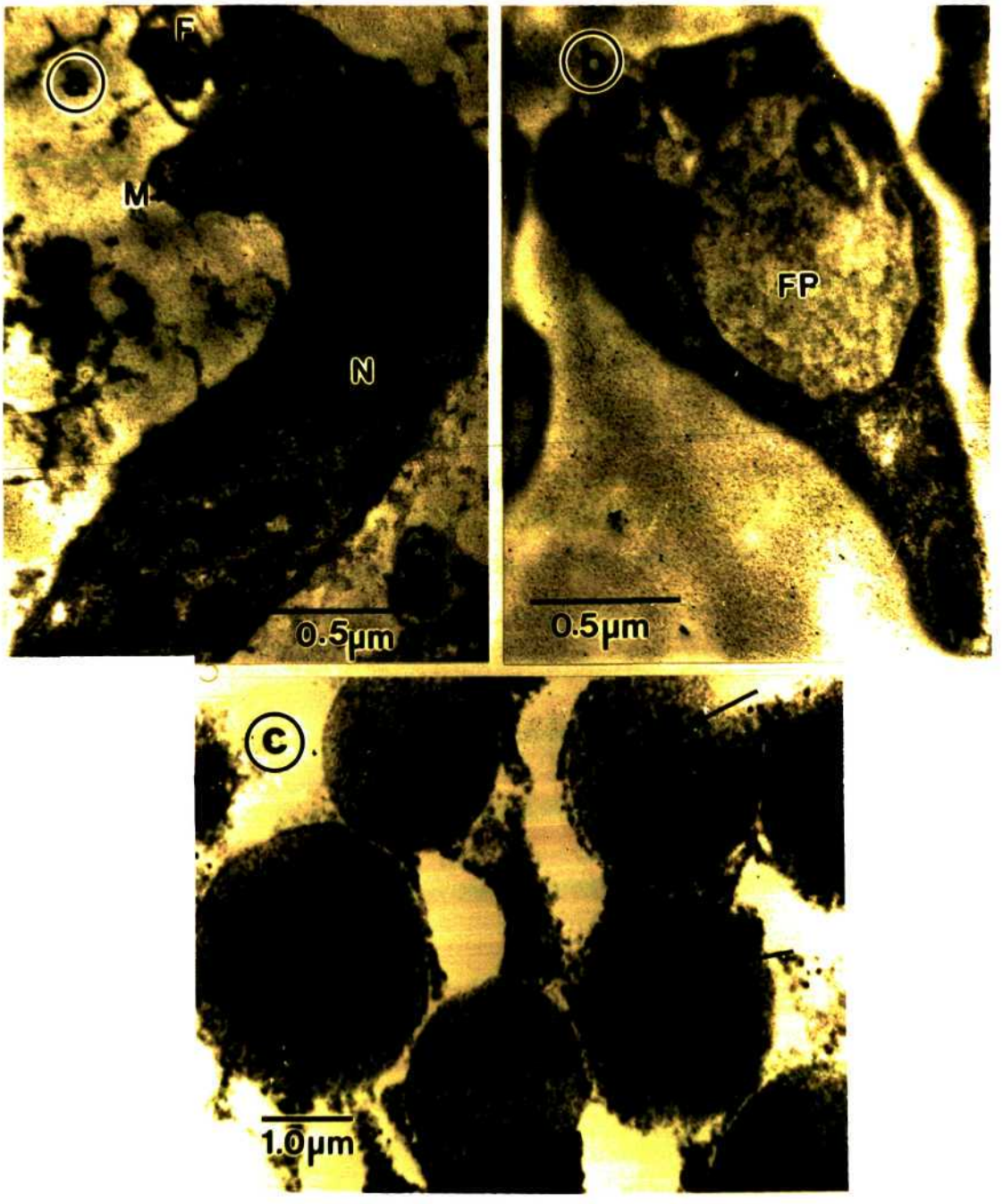
F
M
N
0.5μm
FP
0.5μm
C
1.0μm

Figure 5.3. Transmission electron micrograph of *T brucei* and β-cells of pancreas

(a)– Glutaraldehyde-fixed trypanosomes showing general ultrastructure, Transverse section of the flagella, F, Pellicular microtubules, M, Nucleus, N. (b)– *T. brucei* section incubated with chicken preimmune IgY, rabbit anti-chicken IgY and protein A-gold. (c)–immunocytochemical localisation of glucagon in β-cells of pancreas with anti-glucagon antibodies and protein A-gold conjugates used in the experiments to localise MCP-Tb and VSG in *T. brucei*

5.6 Discussion

Subcellular Localisation

Knowledge of intracellular distribution of MCP is important in elucidating the precise role of the proteinase in the trypanosome. Immunolocalisation of MCP has previously been communicated in a variety of cell types including rat liver, brain and muscle (Palmer *et al.*, 1996, Kamakura *et al.*, 1988), human liver (Tanaka *et al.*, 1989), *Drosophila* (Haass *et al.*, 1989), sea urchin (Akhayat et al., 1987), and newt (Peters *et al.*, 1994). In each of these cases MCP was observed in both the nucleus and cytoplasm. However, there are no reports with regard to intracellular localisation of MCP in trypanosomes.

When MCP-Tb was assayed in the subcellular fractions obtained after differential centrifugation of the *T. brucei* homogenate and the results expressed as relative specific activity, the enzyme represented a soluble cytosolic and organelle-associated distribution of activities. About 22% of the MCP-Tb activity fractionated together with the nuclear fraction containing a large number of cells that resemble those identified by Opperdoes *et al.* (1977a) as ghost cells. Although association of MCP-Tb with the plasma membrane fraction is unlikely, previous reports demonstrated the presence of MCP in the nuclei of eukaryotic cells (Rivett *et al.*, 1992; Palmer *et al.*, 1996). The MCP-Tb activity in the NU fraction presents a pattern similar to malate dehydrogenase activity. The distribution profile of malate dehydrogenase in the present study did not reveal the expected preferential association with mitochondria or glycosomes (Opperdoes *et al.*, 1977a). The mitochondria fractionated mainly in the LG fraction, whereas the glycosomes were reported to be in the SG and partly in the MI fractions (Grab *et al.*, 1987). The distribution of malate dehydrogenase was different from previous reports and could not be reliably used as a marker for an organelle. Although prior evidence for nuclear localisation of MCP is compelling, no conclusion can be drawn from the available results to exclude MCP-Tb association with the nuclear membranes.

From the present results and those in previous reports (Opperdoes *et al.*, 1977a; 1977b), oligomycin-sensitive ATPase is shown to be undoubtedly mitochondrial. The activity of mitochondrial ATPase shows peak activities in the LG with a little trailing in the NU fraction, consistent with the electron microscope confirmation of sedimentation of mitochondria in the LG fraction (Opperdoes *et al.*, 1977a). Considering that 75% of MCP-

Tb activity is in the CY fraction and 22% in the NU fraction, and that maximal specific activity (32%) of mitochondrial ATPase coincides with fractions in which only trace amounts of MCP-Tb are present, the two enzymes are most likely located in different organelles.

Interestingly, cysteine protease and glycerol-3-phosphate dehydrogenase activities presented a similar distribution profile with peak activities in the SG fraction. Cysteine protease is a reliable marker of lysosomes in *T. brucei* (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, 1991b), whereas glycerol-3-phosphate dehydrogenase is located within the glycosomes in *T. brucei* (Opperdoes *et al.*, 1977a; Rovis and Baekkeskov, 1980). Thus lysosomes and glycosomes were probably enriched in the SG fraction of this author's preparation. Since neither cyteine protease nor glycerol-3-phosphate dehydrogenase activity coincides with the F1 or F5 fractions where MCP-Tb is enriched, it is tempting to conclude that neither lysosomes nor glycosomes contain MCP-Tb.

Several marker enzymes have been shown to be latent in organelles and their activities increased several fold by use of detergents such as 0.1% Triton X-100 (Duschak and Cazzulo, 1991; Nowicki *et al.*, 1992; Weitman and Etlinger, 1992). In the present case, MCP-Tb was sufficiently active against the Z-Gly-Gly-Leu-AMC substrate, thus obviating the use of detergent to enhance the proteolytic signals. Part of the reason for high activity of MCP-Tb could be that it exists freely in the cytoplasm rather than within organelles. This enables ease of access of the substrate to the catalytic site of MCP-Tb.

Acid phosphatase had optimal activity in the MI fraction of the present preparation. Acid phosphatase has previous been reported to be localised in the flagellar pocket, lysosomes and Golgi apparatus in trypanosomes (Langreth and Balber, 1975; Grab *et al.*, 1987; Tosomba *et al.*, 1996). Incidentally, the MCP-Tb activity is very low in the MI fraction suggesting that MCP-Tb may not be associated with the flagellar pocket membrane, lysosomes or the Golgi apparatus. The non-lysosomal localisation of MCP-Tb is further supported by enrichment of lysosomal protease (CysP) activity in the SG and LG fractions where there is no significant MCP-Tb activity. The fractionation results indicate that MCP-Tb has a considerable cytosolic activity (75%) and this compares well with the previous reports in sea urchin (Akhayat *et al.*, 1987), human liver (Tanaka *et al.*, 1989) and *Drosophila* (Haass *et al.*, 1989) proteasomes.

Immunocytochemical localisation

Embedding of *T. brucei* cells in Lowicryl K4M resin using the PLT method (Kellenberger, 1985) not only showed good ultrastructural preservation of the cell organelles, but also preserved the epitopes in the *T. brucei* section for successful immunolabeling procedures. Furthermore, the extraction of proteins and membrane lipids from biological materials embedded at low temperature is reduced (Weibull and Christiansson, 1986), and the Lowicryls enabled the use of a low concentration of glutaraldehyde concentration for a short period (Hobot, 1989).

The present investigation of the localisation of the MCP-Tb complex in *T. brucei* using polyclonal antibodies raised against purified trypanosomal MCP shows the presence of MCP in the nuclei and the cytoplasm (Fig 5.1a). These results are consistent with previous reports showing the presence of MCP in the nuclei of mammalian cells (Knetch *et al.*, 1991; Rivett *et al.*, 1992; Palmer *et al.*, 1996), but differ from earlier reports in which no staining of nuclei were observed (Akhayat *et al.*, 1987; Haass *et al.*, 1989, Kamakura *et al.*, 1988; Tanaka *et al.*, 1989). The presence of MCP-Tb in the nuclei and cytoplasm is in agreement with our cell fractionation results showing that MCP-Tb has a high soluble cytosolic activity (Table 5.1) and that the nuclear fraction also has significantly high MCP-Tb activity (Table 5.2). There are discrepancies in the literature with regard to the proportion of MCP in the nucleus and cytoplasm. In some cases the complex has been located largely in the cytoplasm whereas in other cases it appears to be predominantly in nuclei. For example, MCP is predominantly nuclear in growing rat fibroblasts (Arrigo *et al.*, 1988), in human leucocytic and liver cells (Kumatori *et al.*, 1990; Rivett *et al.*, 1992), and in muscle cells (Stauber *et al.*, 1987), but is apparently absent from the nucleus of some human liver cells (Tanaka *et al.*, 1989). It appears that proportions of the nuclear MCP is developmentally regulated such that changes in localisation of MCP is observed during development of lower eukaryotes including *Drosophila* (Haass *et al.*, 1989), sea urchin (Akhayat *et al.*, 1987) and newt (Pal *et al.*, 1988). From the observed strong labeling of the nuclei as compared to the cytoplasm in *T. brucei*, it appears that the proportion of MCP-Tb is higher in the nuclei than the cytoplasm of the bloodstream form of the parasite. However, this difference in labeling may simply be a reflection of different levels of epitope exposure.

The presence of MCP-Tb in the nuclei of *T. brucei* and the reports of intracellular occurrence of polyubiquitin and the ubiquitin-activating enzyme in *T. brucei* (Wong *et al.* 1992, Lowrie *et al.*, 1993), suggests that MCP-Tb may also have a nucleus-specific function in the parasite. Proteolysis may be involved in the regulation of proteins of the nuclear scaffold, which change with the differentiation stage of the cell. The nuclear proteins are believed to play a fundamental role in growth-related functions in the cell nucleus, including regulation of gene expression (Levinger and Varshavsky, 1982). Although localisation of MCP-Tb in the nucleus suggests its function in the degradation of nuclear proteins, it is not certain whether proteins involved in growth-regulation or gene expression are endogenous substrates for the enzyme.

As might be expected from current knowledge, exogenous proteins taken up by endocytosis in trypanosomes are translocated through endocytic vesicles, which eventually fuse with lysosome-like organelles (Mbawa *et al.*, 1991b). Consequently, the proteins are degraded by the lysosomal proteases such as the trypanopains (Lonsdale-Eccles and Grab, 1987; Mbawa *et al.*, 1992). However, considering the nuclear or cytoplasmic localisation of MCP (Rivett *et al.*, 1992; Peters *et al.*, 1994; Palmer *et al.*, 1996), a carrier molecule is envisaged for transmembrane translocation of exogenous proteins from the endocytic vesicles to the cytosol. At the moment no such transporter molecule has been reported putting into question the mechanism by which the exogenous proteins are delivered into the cytosol for degradation by the MCP-Tb. It has been suggested that trypanosomes possess a mechanism for excluding exogenous proteins from part of the endocytosis pathway and that molecular sorting occurs in some of the cisternal organelles associated with the endosomal system of the parasite (Webster and Grab, 1988; Webster and Fish, 1989). The components of the antigen-processing and presentation machinery in mammalian cells may be of relevance in an attempt to elucidate the translocation mechanism of exogenous proteins into the cytosol of the trypanosomes. In mammalian cells, MCP participates in the degradation of cytosolic antigens into short peptides for presentation by MHC class I molecules of antigen presenting cells (Dick *et al.*, 1994; Rock *et al.*, 1994). A transporter molecule (TAP1/TAP2 heterodimer) associated with antigen processing is responsible for the ATP-dependent transmembrane translocations of selected peptides from the cytosol to the site of MHC class 1 assembly (Kleijmeer *et al.*, 1992). A similar transporter molecule may facilitate translocation of materials across organelle

membranes into the cytosol for degradation by MCP-Tb in the parasite (Wilbourn *et al.*, 1998).

VSG is known to cover the surface of the parasite, including the flagellum and the flagellar pocket (Vickerman, 1969; Cross, 1990). Thus, immunolocalisation of VSG at these sites served as a control for the methodology. It is notable that localisation of VSG and MCP-Tb in the same intracellular compartment may not necessarily have a functional meaning. For instance, VSG is synthesised on the ribosomes and the polypeptide enters the lumen of the rough endoplasmic reticulum and is transported to the smooth endoplasmic reticulum and Golgi apparatus where post-translational modification takes place (Grab *et al.*, 1984; Ferguson *et al.*, 1988; Cross, 1990). MCP has been shown to be responsible for processing of several ER-proteins (Coux *et al.*, 1996), however, not all ER-proteins may be substrates for the MCP. In the present case, MCP-Tb was shown to be cytosolic but its association with ER is not evident. Nevertheless, previous reports indicate that MCP is localised on the cytoplasmic side of the ER, in addition to the cytoplasm and nucleus (Rivett *et al.*, 1992; Peters *et al.*, 1994). Since lumenal ER-proteins are thought to translocate across ER membrane to the cytoplasmic side for proteasomal processing (Wilbourn *et al.*, 1998), this ER-associated MCP may participate in the degradation of ER-proteins.

Interestingly, recent evidence shows a direct implication of MCP in quality control of glycophosphatidylinositol (GPI) anchor addition to proteins (Wilbourn *et al.*, 1998). VSG vary biochemically and thus antigenically, however, one part of the VSG molecule stays relatively constant (Zamze *et al.*, 1988). This part, called a 'cross-reacting determinant' (CRD) is a complex glycolipid side-chain structure of the VSG molecule, identified as GPI, believed to anchor VSG on to the plasma membrane of African trypanosomes (Englund, 1993). Inhibition of MCP activity by lactacystin blocks the intracellular processing ofprecursor GPI suggesting involvement of MCP in GPI quality control (Oda *et al.*, 1996). Improperly processed GPI-anchored proteins are degraded by ER-associated MCP and involve re-export of lumenal proteins into the cytoplasm for ultimate degradation by the MCP (Wilbourn *et al.*, 1998). Thus, it is our hypothesis that inhibition of MCP-Tb activity in trypanosomes may interfere with the synthesis and addition of the GPI-anchor of the trypanosomal VSG. Chymostatin, an inhibitor of chymotrypsin-like activity in MCP-Tb, was reported to inhibit clearance of bound anti-VSG antibodies on the surface of *T. brucei* (Russo *et al.*, 1993). However, this phenomenon may be ascribed more to the

endocytotic role of MCP (Staub *et al.*, 1997; Strous and Govers, 1999) rather than the interference with post-translational processing of the GPI-anchored protein (Oda *et al.*, 1996; Wilbourn *et al.*, 1998) in the parasite.

Lastly, nuclear localisation of MCP-Tb (Fig. 5.1b) suggests a possible role of MCP-Tb in the control of gene expression. There are several genes coding for VSGs that are expressed sequentially in a mutually exclusive manner (Borst and Cross, 1982). The synthesis of VSG is under strict transcriptional control in the parasite (Steinert and Pays, 1985). MCP may exert its transcriptional control over VSG synthesis by degradation of nuclear proteins involved in gene expression (Levinger and Vershavsky, 1982). Thus, it appears reasonable to conclude that inhibition of MCP-Tb may interfere with the synthesis of VSG. The problem as it were is the success of inhibition of the parasite MCP-Tb without significantly affecting the host cell MCP and the relevant physiological processes of the host.

In conclusion, the antibodies produced as described in the previous chapters to specifically target MCP-Tb and VSG in *T. brucei* sections, proved useful in showing the intracellular distribution of MCP-Tb in the parasite. The surface associated labeling of VSG was expected and served to validate the methodology used to localise MCP-Tb in the present study. The cytosolic and the nuclear localisation of MCP-Tb in the present study is consistent with literature reports of the localisation of a similar enzyme in other cell-types, providing circumstantial evidence for its role in cytosolic and nuclear proteolysis. The next chapter presents a study on how these proteolytic activities of MCP-Tb could be enhanced by interaction with the host regulatory molecule, PA700.

Chapter 6

Studies on the effects of bovine PA700 on MCP-Tb

6.1 Introduction

The proteasome is a cylindrically shaped, intracellular complex of proteins. Its proteolytic activity is modulated by a number of specific regulatory proteins. One such regulator is called PA700 or 19S regulator. This is a protein complex that has no proteolytic activity by itself. However, it activates multiple proteolytic activities of the 20S proteasome. The active sites (threonine residues) of proteasomes are located within the interior of the proteasomes and access to these sites seems to be regulated by large (19S and 11S) protein complexes (e.g. Coux *et al.*, 1996; Tanaka, 1998) that appear to cap the ends of the cylinders. Association of the proteasome with either of these caps greatly enhances the hydrolysis of fluorogenic peptides (Chu-Ping *at al.*, 1992; 1994). Complexes of the 20S proteasome with the 11S particle (also called PA28) can degrade peptides only. Thus, the 20S/11S complex particles have no effect on protein degradation and do not use adenosine 5'-triphosphatase (ATP) for activation (Chu-Ping *et al.*, 1994). In contrast, the complexes of the 20S proteasome with the 19S particle (also called PA700; the term used in this study) called the 26S proteasome can degrade both peptides and proteins, particularly ubiquitinated proteins, in an ATP dependent manner (DeMartino *et al.*, 1994; Tanaka, 1998). Interestingly, a recent report indicates that the 20S proteasome can bind both PA700 and PA28 simultaneously to form an even larger ternary complex (Hendil *et al.*, 1998), but the significance of this is not clear.

PA700 is a 700 kDa protein complex composed of approximately 16 different subunits (Chu-Ping *at al.*, 1994). The masses of the usbunitsrange between 25 and 110 kDa. Six of these subunits belong to the rapidly growing ATPase family of proteins called AAA (ATPase Associated to a variety of cellular Activities) (Confalonieri and Duguet, 1995; Coux *et al.*, 1996; Lupas *et al.*, 1997).

Although 26S proteasomes have been isolated from several eukaryotic organisms (Tanaka, 1998), it is not clear whether trypanosomes have a 26S proteasome. Indeed the failure to isolate an intact 26S complex from these organisms led To and Wang (1997) to conclude that

trypanosomes may not possess an ATP-ubiquitin-dependent pathway of protein degradation. However, several observations tend to argue against this conlusion, including: the probable involvement of ubiquitin-like processes in the degradation of high turnover *T. cruzi* proteins (Henriquez *et al.*, 1993); the presence of several polyubiquitin genes in *T. brucei* (Wong *et al.*, 1992); and the presence of various components of the ubiquitin-ligase system in different trypanosomatids including *T. brucei* (Lowrie *et al.*, 1993). Furthermore, in this Chapter, evidence is provided showing that bovine PA700 enhances the activity of the *T. b. brucei* 20S proteasome in an ATP dependent manner. Hence we may infer that the ATP-dependent pathway of protein degradation is present in trypanosomes and that the failure thus far to identify a 26S proteasome (or the PA700-like molecule) in trypanosomes may be due to an unusual lability of this complex in trypanosomes.

6.2 Purification of bovine PA700

PA700 was purified from bovine red blood cells as described by Chu-Ping *et al.* (1994). The PA700 co-purifies with the bovine 20S proteasome and PA28 activator in most stages of purification. However, separation of PA700 from the 20S proteasome and PA28 may be achieved by ammonium sulphate precipitation. The bovine PA700 protein complex usually precipitates from a clear fraction (DEAE-fractionated) at 38% ammonium sulphate saturation whereas the 20S proteasome remains in solution at this ammonium sulphate concentration. Proteasomes require glycerol (20% v/v) to maintain enzyme stability (McGuire *et al.*, 1988) whereas PA700 does not (Chu-Ping *et al.*, 1994), hence the need to use buffer G (Section 3.2) for purification of the proteasome.

5.2.1 Reagents

Phosphate buffered saline (PBS). See Section 2.12.1

Standard buffer H. See Section 3.2

Equilibrating buffer (Standard buffer H containing100 mM NaCl). NaCl (5.844 g) was added to 950 ml of standard buffer, adjusted to pH 7.6 with HCl and made up to 1 litre with standard buffer H.

Elution buffer (Standard buffer H with additional 500 mM NaCl). NaCl (29.22 g) was added to 950 ml of standard buffer, adjusted to pH 7.6 with HCl and made up to 1 litre with standard buffer H.

Sodium phosphate buffer (20 mM Na-phosphate buffer, pH 7.6). $NaH_2PO_4.2H_2O$ (3.13 g) was dissolved in 950 ml of dist. H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre.

Sodium phosphate elution buffer (200 mM Na-phosphate, pH 7.6). $NaH_2PO_4.2H_2O$ (31.2 g) was dissolved in 950 ml of dist. H_2O, titrated to pH 7.6 using NaOH, and made up to 1 litre.

6.2.2 Procedure

A portion of the crude bovine red blood cell lysate, "Fraction I" (Section 3.3.2) was used for the isolation of the proteasome activator PA700. DE-52 cellulose was equilibrated with standard buffer H containing 100 mM NaCl. The DEAE cellulose resin was added to the red blood cell lysate supernatant (approximately 5 ml of lysate per ml of DE-52) and mixed for 30 min. The resin was filtered and washed extensively until the eluate was clear. The resin was mixed with elution buffer (1 ml elution buffer/ml DE-52), stirred gently for 10 min, filtered and washed with additional volumes of elution buffer until most of the bound proteins were eluted. The clear fraction eluted was termed "Fraction II" (Chu-Ping *et al.*, 1994).

Ammonium sulfate was added to Fraction II to 38% saturation over 30 min with constant stirring. Precipitated proteins were collected by centrifugation. The pellet was resuspended in a large volume of standard buffer H, saturated to 38% ammonium sulfate. The pellet was washed in buffer H saturated to 38% ammonium sulfate to remove PA28 and the 20S proteasome, both of which are soluble at this salt concentration. The pellet was dissolved in a small volume of equilibrating buffer and dialyzed for 16 h against a large volume of the same buffer. After dialysis, insoluble material was removed by centrifugation (30 000 x g; 30 min) and the soluble fraction subjected to chromatography on Sephacryl S-300 using equilibrating buffer.

Fractions collected were assayed for PA700 activity (Section 6.2.4). Fractions containing the highest level of activity were pooled and subjected to ion exchange chromatography on DEAE-Toyopearl (2.5 x 10 cm) equilibrated (30 ml. h^{-1}) with equilibrating buffer. Bound proteins were eluted with a linear gradient of 100–350 mM (400 ml) NaCl in equilibrating buffer. Eluted fractions were assayed for PA700 activity. Active fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, pH 7.6, and applied to HA-Ultrogel (1.5 x 12 cm) equilibrated (20 ml. h^{-1}) in the same buffer. PA700 was eluted by a linear gradient of 20–200 mM phosphate buffer. The active fractions were concentrated by ultrafiltration and stored at –70°C.

6.2.3 Phosphatase assay

Assays for phosphatase activity of purified PA700 were based on the ATPase assays of Reenstra and Forte (1990). This assay uses *p*-nitrophenyl phosphate as substrate. Upon hydrolysis, the *p*-nitrophenyl phosphate liberates inorganic phosphate and *p*-nitrophenol. The substrate is colourless, but the product *p*-nitrophenol is yellow in alkaline solution, absorbing maximally at 405 nm. Hence progress of this reaction may be followed continuously at this wavelength using a spectrophotometer.

6.2.3.1 Reagents

Assay buffer (20 mM Hepes/Tris buffer, pH 7.2). Hepes (476.6 mg) was dissolved in 80 ml of dist. H_2O, adjusted to pH 7.2 with solid Tris and made up to 100 ml.

Para-nitrophenyl phosphate [50 mM Na_2-pNPP]. See Section 5.3.1

Stopping solution (10 mM NaOH). See Section 5.3.1

6.2.3.2 Procedure

The reaction mixture consisted of purified PA700 (1–2 µg), assay buffer, pH 7.2 (50 µl), 50 mM *p*-nitrophenyl phosphate (10 µl), 2 mM ouabain (5µl), with or without of 100 mM KCl (10 µl), in a total volume 100 µl. When required, vanadate was dissolved in the assay buffer at the required concentration. Incubations were carried out for 60 min at 37°C with gentle shaking (50 oscillations per minute). The reactions were stopped by the addition of 20 mM NaOH (50 µl). The amount of *p*-nitrophenol liberated was determined spectrophotometrically at 405 nm using a BIO-TEK EL 312 microtitre plate reader.

Potassium ion-stimulated phosphatase activities were determined from the difference in optical density in the presence and absence of K^+. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmole *p*-nitrophenol per minute under the above conditions. An extinction coefficient of 1.465 $\mu mole^{-1}.ml.cm^{-1}$ was used to determine concentration of released *p*-nitrophenol (Tosomba *et al.*, 1996).

6.2.4 Activation Studies

6.2.4.1 Reagents

Assay buffer [90 mM Tris, 10 mM DTT, 0.12 mM ATP, 20 mM $MgCl_2$, pH 8.0]. Tris (2.725 g), DTT (0.386 g), ATP (0.0165 g) and $MgCl_2$ (1.017 g) were dissolved in 200 ml of dist.H_2O, adjusted to pH 8.0 with HCl and made up to 250 ml with dist.H_2O.

Substrate stock solution. See Section 3.4.1.1

6.2.4.2 Procedure

Purified MCP-Tb and PA700 were pre-incubated at 37°C for 45 min essentially as described (Chu-Ping *et al.*, 1994). This pre-incubation mixture consisted of assay buffer (50 μl), purified MCP-Tb (0.2 μg) and PA700 (1-2 μg) in a final volume of 75 μl. After the pre-incubation, 25 μl of substrate (50 μM peptide-AMC or 0.2 mM peptide-βNA final conc.) in 30 mM Tris/HCl, pH 8.0 containing 1 mM dithiothreitol was added and the release of AMC or βNA was measured spectrophotometrically (Section 3.4.1.2). Control assays, conducted in parallel, consisted of pre-incubation mixtures and subsequent assay mixtures in the presence and absence of proteasome and activator.

To study the effect of the activator on the kinetic properties of the enzyme, MCP-Tb (0.2 μg) was pre-incubated with ATP and saturating amounts of activator (2 μg). The activity of the MCP-Tb was assayed against increasing concentrations of each peptide-AMC substrate (from 0-500 μM) or Z-Leu-Leu-Glu-βNA (0-2 mM). Control experiments contained no PA700 activator. The maximal reaction velocity (V_{max}) and substrate concentration required for half-maximal velocity (apparent K_m) were estimated by hyperbolic regression using the software package Hyper (obtained from Dr. J. S Easterby, University of Liverpool, U.K.).

6.2.5 Assay for PA700-Proteasome Association

Under physiological conditions, the proteasome exists as a 20S complex or, when in combination with the PA700 activator, as a 26S proteasome (Coux *et al.*, 1996). The 26S may be isolated intact from mammalian cells using gentle protein purification techniques (Dahlmann *et al.*, 1995). However, dissociated 20S proteasome and its PA700 activator may also be isolated separately and may then be associated *in vitro* (Chu-Ping *et al.*, 1994). This section describes an assay to measure the association of trypanosomal MCP-Tb and PA700 isolated from bovine red blood cells.

6.2.5.1 Reagents

Phosphate buffered saline (PBS), pH 7.4. See Section 2.12.1

BSA-PBS [0.5% (w/v) BSA-PBS]. See Section 2.12.1

PBS-Tween [0.1% (v/v) Tween 20/PBS]. See Section 2.12.1

10 mM Tris/HCl, pH 7.5. Tris (1.21 g) was dissolved in 950 ml of dist. H_2O, titrated to pH 7.5 with HCl and made up to 1 litre.

Tris/HCl-Tween [0.1% Tween 20 (v/v)/Tris-HCl, pH 7.5]. Tween 20 (1 ml) was made up to 1 litre with stirring in 10 mM Tris-HCl, pH 7.5.

Elution buffer (20 mM Tris-HCl, 0,5 mM NaCl, pH 7.5). Tris (2.42 g) and NaCl (0.029 g) were dissolved in 950 ml of dist. H_2O, titrated to pH 7.5 with HCl and volume made up to 1 litre with dist. H_2O.

Antibodies. Anti-PA700 IgY and anti-proteasome IgY were raised in chickens and isolated as described in Section 2.10.3. Antibodies were raised in rabbits against chicken IgY and isolated as described in Section 2.10.4.

6.2.5.2 Procedure

A direct binding assay, adapted from Realini *et al.* (1997) was used to determine whether bovine PA700 binds the MCP-Tb isolated from *T. b. brucei*. The wells in a microtiter plate were coated (16 h; 4°C) with 150 µl of rabbit anti-chicken IgY at 20 µg/ml in PBS.

Unoccupied sites in the wells were blocked with 200 µl of 0.5% (m/v) BSA-PBS by incubation for 1 h at 37°C. The microtitre plate was washed three times between each incubation step with PBS-Tween. This was followed by incubation with chicken anti-trypanosomal MCP-Tb IgY or control pre-immune IgY at concentrations of 20 µg/ml in 0.5% BSA-PBS (200 µl) for 2 h at 37°C. Trypanosomal MCP-Tb (200 µl, 30 µg/ml in BSA-PBS) was incubated in each well for 16 h at 4°C followed by incubation with various dilutions of PA700 in 10 mM Tris/HCl, pH 7.5 for 20 min, at room temperature and 120 min at 4°C. The plates were quickly rinsed twice with an excess of cold (4°C) 10 mM Tris/HCl, pH 7.5, PBS-Tween and once with 10 mM Tris/HCl, pH 7.5. The bound PA700 was eluted with 150 µl of 0.5 M NaCl in 20 mM Tris/HCl, pH 7.5, and dot-blotted onto nitrocellulose for probing with chicken anti-PA700 IgY. Bound antibodies were detected as described (Pike *et al.*, 1992) using alkaline phosphatase-conjugated rabbit anti-chicken IgG that cross-reacts with chicken IgY and BCIP/NBT substrate.

6.3 Results

6.3.1 Subunit analysis

Both the bovine-derived proteasome activator PA700 and the trypanosome-derived MCP-Tb were purified to apparent homogeneity as evidenced by single bands on native PAGE gels (Fig. 6.1A; lanes 2 and 3 respectively). The molecular masses of PA700 and the MCP-Tb were each estimated by molecular exclusion chromatography to be about 700 kDa. Since PAGE separates proteins on the basis of their charge-to-mass ratio, the molecular mass indicated in Fig 6.1 A is merely a rough estimation and consequently lower than that indicated by molecular exclusion chromatography. When the bovine PA700 was subjected to reducing SDS-PAGE, multiple protein bands ranging from 20–100 kDa were observed (Fig. 6.1B; lane 1). This protein profile is similar to that reported before (DeMartino *et al.*, 1994). Under similar conditions, the trypanosomal MCP-Tb showed protein bands ranging from 18-27 kDa (Fig. 6.1B; lane 2). This profile is characteristic of eukaryotic proteasomes (Tanaka, 1998) and differs from that of prokaryotes (Löwe *et al.*, 1995). There was no difference in the subunit profiles of PA700 and the MCP-Tb under reducing and non-reducing SDS-PAGE conditions (results not shown).

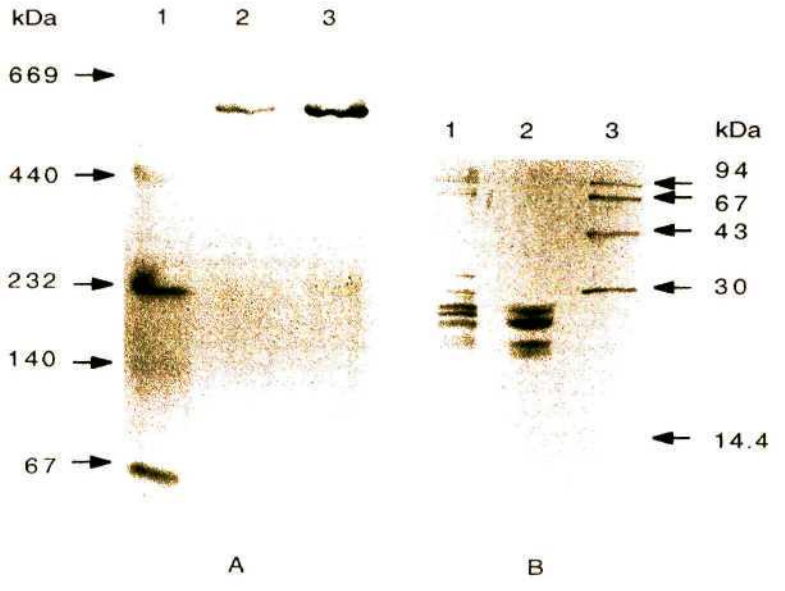

Figure 6.1. Electrophoretic analyses of purified PA700 and MCP-Tb

Panel A, non-denaturing PAGE of M_r markers (lane 1) as in legend to Fig. 3.4, PA700 (lane 2) and MCP-Tb (lane 3). *Panel B*, reducing SDS-PAGE (12.5%) analysis of PA700 (lane 1); proteasome (lane 2); M_r markers (lane 3) as in legend to Fig. 3.4.

6.3.2 Specificity of anti-MCP-Tb and anti-bovine PA700 antibodies

To assess recognition of the trypanosomal 20S proteasome and bovine PA700, by their respective antibodies, immunoblots of the native proteins (Fig. 6.2, *panel A*) and their respective subunits (Fig. 6.2, *panel B*) were prepared. Chicken anti-PA700 recognised intact bovine PA700 (Fig. 6.2A; *lane* 1) and two of its larger subunits of 97 and 104 kDa (Fig. 6.2B; *lane* 1) but did not recognise the other subunits. There was no cross-reactivity between anti-PA700 antibodies and any of the protein bands of trypanosome lysates separated on native PAGE (results not shown). Chicken anti- trypanosomal proteasome antibodies recognised the trypanosomal 20S proteasome on blots of native PAGE gels (Fig. 6.2A; *lane* 2) and only some of its subunits (in the region of 27 kDa) on blots of SDS-PAGE gels (Fig. 6.2B; *lane* 2). The anti-20S proteasome antibody did not cross-react with bovine PA700 or any of its subunits (results not shown).

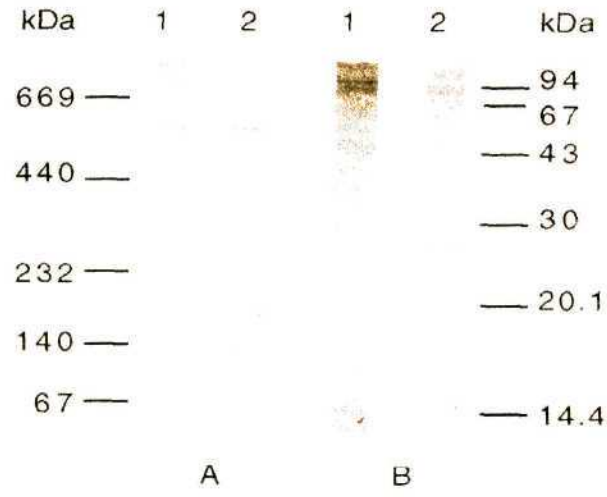

Figure 6.2. Immunoblot analysis of PA700 and the MCP-Tb

Purified PA700 (lane 1) and MCP-Tb (lane 2) were electroblotted from non-denaturing PAGE (*Panel A*) and reducing SDS-PAGE (*Panel B*) onto nitrocellulose and probed by chicken anti-PA700 IgY (*Panel A*, lane 1 and *Panel B*, lane 1) and chicken anti-proteasome IgY (*Panel A*, lane 2 and *Panel B*, lane 2) antibodies. The bound antibodies were detected as described under Section 6.2.5. The positions of the M_r markers are indicated.

6.3.3 Phosphatase activity of PA700

Bovine PA700 *p*NPPase activity was activated by K^+ ions in a concentration dependent manner (Fig. 6.3).

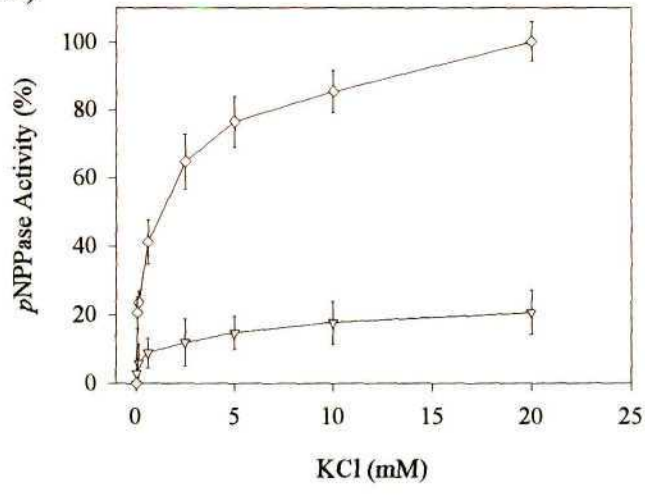

Figure 6.3. Effect of K^+ ion concentration on *p*-nitrophenyl phosphatase (*p*NPPase) activity of PA700.

Reaction mixture (100 μl) contained 20 mM Hepes/Tris buffer, pH 7.2 and either 2 μg (◊) or 0.2 μg of PA700 (▽), with the indicated KCl concentrations. Reactions were initiated by the addition of 5 mM *p*-nitrophenyl phosphate. The results are presented as means ± S.E of three independent experimental determinations.

The *p*NPPase activity was not detectable in the absence of K^+ ions and only became detectable at K^+ concentrations above 0.2 mM. The maximum catalytic activity was observed at 5 mM K^+ (for 2 μg PA700/100 μl) and 0.5 mM K^+ ion (for 0.2 μg PA700/100 μl). The K^+ ion concentration required for activation is in the same range as that reported for *p*NPPase activities of gastric oxyntic cells (Reenstra and Forte, 1990).

6.3.4 Effect of bovine PA700 on the activity of trypanosomal MCP-Tb

Bovine PA700 increased the activity of the trypanosomal 20S proteasome against Suc-Leu-Leu-Val-Tyr-AMC in a dose-dependent manner from 8 to 21 nmol.min.$^{-1}$mg^{-1} (Fig. 6.4).

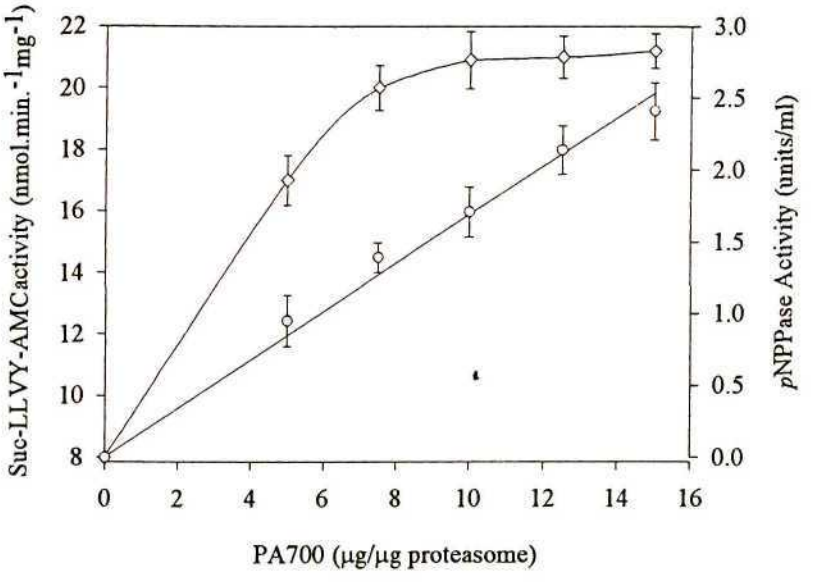

Figure 6.4. Effect of PA700 concentration on *p*NPPase activity and on the hydrolytic activity of the 20S proteasome

Proteasome (0.2 μg), preincubated with increasing amounts of PA700 activator (0–3 μg), was assayed against Suc-Leu-Leu-Val-Tyr-AMC (50 μM;◊). *p*NPPase activity of PA700 was assayed against *p*NPP (5mM;O). Results are presented as means ± S.E of three independent experiments

Maximal activation was reached at 10 μg PA700/μg proteasome (final concentration in the assay mixture). About 3.5 μg PA700/μg proteasome caused 50% activation of proteasome activity (to ~14.5 nmol.min.$^{-1}$mg^{-1}). Maximal velocity was reached at a proteasome:activator ratio of 1:10 (mol:mol). Saturation of the stimulatory effect by PA700 indicated that there was no contamination of the activator preparation with bovine erythrocyte proteasome. The *p*NPPase activity increased linearly with an increase in

concentration of PA700 (Fig. 6.4). PA700 incubated without proteasome did not show hydrolytic activity against Suc-Leu-Leu-Val-Tyr-AMC.

6.3.5 Effect of PA700 on the kinetics of MCP-Tb hydrolysis

The kinetic parameters of the proteasome-catalysed hydrolysis of different substrates in the presence and absence of PA700 are presented in Table 6.1. The chymotrypsin-like, trypsin-like and peptidyl glutamylpeptide hydrolase activities of the trypanosomal proteasome were determined using Suc-Leu-Leu-Val-Tyr-AMC, Z-Gly-Gly-Arg-AMC and Z-Leu-Leu-Glu-βNA, respectively. PA700 enhanced the proteasome activities by increasing the apparent V_{max} (maximal reaction velocity) and by decreasing the apparent K_m (Michaelis constant). In each case, the increase in activity (as assessed by the second order rate constants) was about 5–6 fold. No enhancement of proteasome activity was observed in the absence of ATP.

Table 6.1. Effect of the activator PA700 on the catalytic properties of the trypanosomal MCP-Tb. Activator protein was purified from bovine red blood cells as reported in Section 5.2.2. Proteasome purified from *T. b. brucei* was preincubated in the absence and presence of saturating amounts of the activator. The proteasome activity was assayed with increasing concentrations of peptide substrates; Suc-Leu-Leu-Val-Try-AMC (0-500 μM), Z-Gly-Gly-Arg-AMC (0-500 □M), Z-Leu-Leu-Glu-βNA (0-2 mM) and values for the apparent K_m (μM) and V_{max} (nmol.min^{-1}.mg^{-1}) estimated by hyperbolic regression using a software package Hyper. The data presented are mean values ± S.E of three independent experimental determinations.

	-PA700			+PA700			V_{max}/K_m
Peptide Substrates	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	**ratio ± S.E**
Suc-Leu-Leu-Val-Try-AMC	7.98 ± 0.63	81 ± 5	0.099	26.2 ± 2.6	59 ± 6	0.444	4.5 ± 0.14
Z-Gly-Gly-Arg-AMC	3.57 ± 0.23	79 ± 3	0.045	10.5 ± 0.9	40 ± 4	0.263	5.8 ± 0.09
Z-Leu-Leu-Glu-βNA	0.86 ± 0.08	235 ± 34	0.004	3.1 ± 0.5	138 ± 16	0.022	6.0 ± 0.21

6.3.6 Binding of PA700 to trypanosomal 20S proteasome

The fact that bovine PA700 stimulates proteasome-catalysed peptide hydrolysis suggests that PA700 is capable of binding to trypanosomal proteasomes. To test this, we performed binding experiments between PA700 and the proteasome (Fig. 6.5). Rabbit anti-chicken IgY antibodies were adsorbed to microtitre plates to serve as capture antibody for chicken anti-proteasome IgY. The anti-proteasome antibodies subsequently bound proteasome without any obvious impediment. In turn, the immobilised proteasome specifically interacted with PA700 (Fig. 6.5). Subsequent detection with anti-PA700 antibodies on dot blots showed that it was possible to dissociate PA700 from the proteasome by using high

salt concentrations. The similar intensity (Fig. 6.5, column *a*) of dot blots having different concentrations (40–4 μg/ml) of PA700 may be interpreted to mean that the proteasome and PA700 bind strongly to each other. Virtually no PA700 was present in samples obtained from wells where pre-immune IgY was used instead of anti-proteasome antibodies (Fig. 6.5, column *b*), or where anti-proteasome antibodies were present but no proteasome was added (Fig. 6.5, column *c*). The latter observation implies that anti-proteasome antibodies immobilised in the microtiter wells were specific for proteasome and did not cross react with PA700. The specificity of anti-proteasome antibodies for its antigen was confirmed by immunoblot analysis (Fig. 6.2).

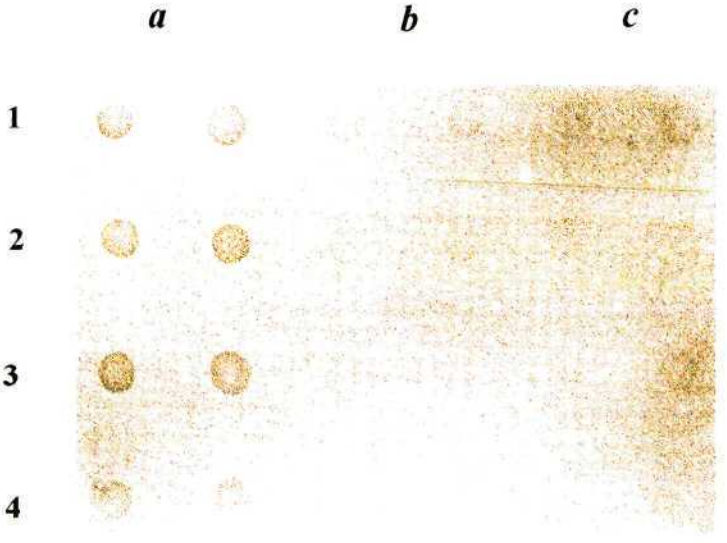

Figure 6.5. Binding assay for PA700-proteasome interaction

MCP-Tb immobilised in the wells of a microplate was incubated with either anti-proteasome antibodies (column *a*) or pre-immune IgY (column *b*). Column *c* had no MCP-Tb (see details under Section 6.2.5). This was followed by incubation with 40 (row 1), 20 (row 2), 10 (row 3), 4 (row 4) μg/ml of PA700 regulator protein. The bound PA700 proteins were eluted with high salt buffer and dot-blotted in duplicate onto nitrocellulose (columns *a*– *c*; rows 1–4). Presence of PA700 on the nitrocellulose membrane was probed with chicken anti-PA700 IgY and detected by rabbit anti-chicken alkaline phosphatase conjugate and BCIP/NBT substrate.

The slight precipitation of proteins in row one of column *b* and *c* is interpreted as due to non-specific interaction between the proteins at high concentration (Fig. 6.5). Duplicate blots incubated with pre-immune IgY preparations instead of anti-PA700 antibodies were negative (results not shown).

6.3.5 Effect of phosphatase inhibitors on PA700 activity

Ouabain (0–125 μM) had no significant effect on the *p*NPPase activity of PA700 (Fig. 6.6). A slight change (corresponding to a change of about 5% from the original activity) may be due to non-specific interactions between ouabain and PA700. In contrast, sodium vanadate inhibited the *p*NPPase activity strongly with an IC_{50} of about 18 μM (Fig.6.6)

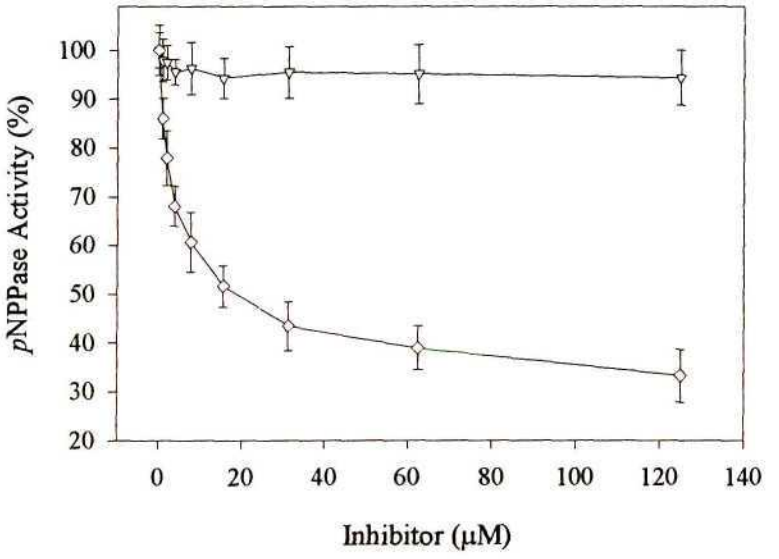

Figure 6.6. Effect of vanadate and ouabain on *p*NPPase activity of PA700.

The PA700 activator (2 μg) was preincubated for 45 min at 37°C with increasing concentrations of vanadate (◇) and ouabain (▽) (0-120 μM). Residual activity against *p*NPP was determined as outlined in Section 3.2.3. The results are presented as means ± S.E for three independent experiments.

Furthermore, vanadate blocked the PA700-induced enhancement of protease activity. This was observed with each of the protease substrates tested (Fig. 6.7). In the absence of vanadate, a 1:5 molar ratio of proteasome: PA700 (required for half-maximal activation) increased the activity (V_{max}) against Suc-Leu-Leu-Val-Tyr-AMC 2.1-fold, Z-Gly-Gly-Arg-AMC 1.6-fold and Z-Leu-Leu-Glu-βNA 2.3-fold. However, none of these proteasomal activities was stimulated by PA700 when 20 μM sodium vanadate was present (Fig. 6.7), or when ATP was absent from the reaction mixture.

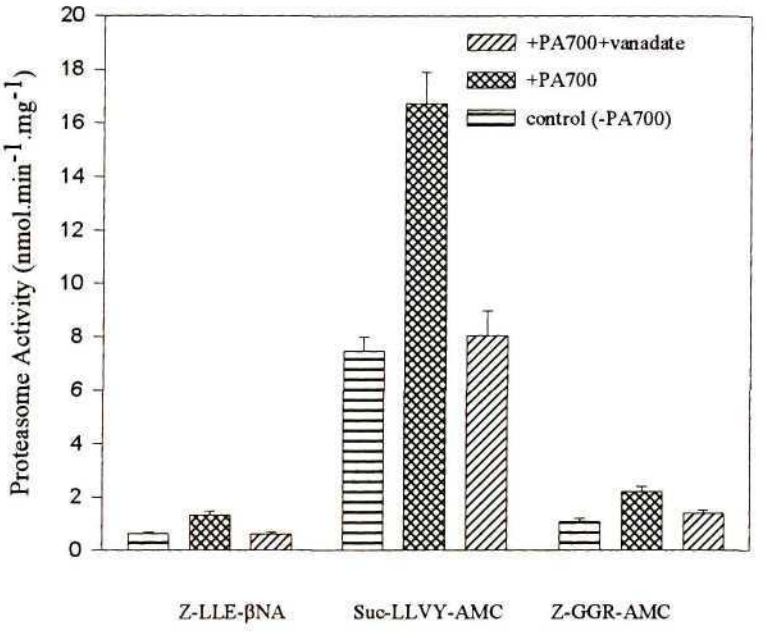

Figure 6. 7. Effect of vanadate on PA700 activation of MCP-Tb

Purified proteasome (0.2 μg) was incubated for 45 min at 37°C with PA700 (1 μg) in the absence or presence of vanadate (20 μM). Control experiments contained no PA700 or vanadate. Proteasome activity was assayed against Suc-Leu-Leu-Val-AMC (50 μM), Z-Gly-Gly-Arg-AMC (50 μM) and Z-Leu-Leu-Glu-AMC (0.2 mM). The fluorescence resulting from the hydrolysis of the substrates was determined as described under Section 3.4.1. Plots show initial velocity (nmol.min.$^{-1}$.mg^{-1}) ± S.E of the means from three independent experimental determinations.

6.4 Discussion

Proteasomes are multimeric, threonine-proteinases that are found in eukaryotic and prokaryotic cells. Two classes of eukaryotic proteasomes (called 20S and 26S) have been identified and these may be isolated from the same cell (Tanaka, 1998). The 26S proteasome is composed of the 20S proteasome and a 19S particle called PA700. Whereas the 20S proteasome does not require ATP for activity (Chu-Ping *et al.*, 1994; DeMartino *et al.*, 1994), the 26S proteasome does require ATP (approximately 60 μM) for activity. The bovine 20S proteasome is activated by as much as 15–100-fold when PA700 is added, with half-maximal activation achieved at a proteasome:activator molar ratio of about 1:3 (Chu-Ping *et al.*, 1994). In this chapter, we report a 5–6-fold activation of the MCP-Tb by bovine PA700 at a 1:10 proteasome:activator molar ratio. These differences may be

attributed to differences between the activators and proteasomes from different species. Nevertheless, the important point is that bovine PA700, a component of the 26S proteasome, is able to activate the trypanosomal MCP-Tb. Such cross-species activation was also seen in the activation of lobster 20S proteasome by bovine PA28 (Mykles, 1996). In contrast to our studies, however, the activation of the lobster proteasome varied from 0–99-fold depending on the substrate studied.

Like the bovine 20S proteasome, the trypanosomal MCP-Tb only showed PA700-enhanced protease activity when ATP was present. Apart from showing that ATP is a necessary component of this activation process, this observation confirmed that the PA700 preparation was not contaminated with another proteasome activator such as PA28. The need for ATP in the PA700–MCP-Tb activation process is also supported by the inhibition of both the *p*NPPase activity of PA700 itself, and the enhancement of MCP-Tb activity in the presence of PA700 by sodium vanadate (Fig. 6.7). One proposed role of the ATPase is to supply energy continuously for the selective degradation of target proteins by the active 26S proteasome (Coux *et al.*, 1996). This energy is presumed to be used in the unfolding of substrate proteins to allow them to penetrate the channel of the α- and β-rings of the 20S proteasome (Tanaka, 1998). However, peptide substrates were used in the present experiments, hence peptide unfolding seems an unlikely scenario for the enhancement observed in this study.

Thus far, neither a PA700-equivalent, nor an intact 26S proteasome, has been reported to occur in African trypanosomes. However, a molecule equivalent to PA28 (named PA26) which enhances trypanosomal proteasome activity, has been isolated from *T. b. brucei* (To and Wang, 1997). Thus, at least one component of the proteasome regulatory mechanism is present in these parasites. The present observation of the binding and enhancement of trypanosomal MCP-Tb activity by bovine PA700 is consistent with the established binding of bovine PA700 to the bovine 20S proteasome (for review see Coux *et al.*, 1996). This observation leads us to suggest that the general mechanism(s) of activation of the trypanosomal MCP-Tb by PA700 is similar to that found in other eukaryotic cells. Consequently, it seems likely that an isoform of PA700 is likely to be present in trypanosomes.

The interaction between PA700 and the 20S core of the 26S proteasome from *T. b. brucei*

is possibly weaker than that of other organisms, resulting in the dissociation of the complex into its regulatory and catalytic sub-complexes during the purification process. Alternatively, the trypanosome equivalent of PA700 may be sensitive to proteolytic digestion. Considering the apparent tight binding between PA700 and the MCP-Tb, and the reaction, or lack thereof, of anti-PA700 antibodies with trypanosome lysates, the latter interpretation seems to be the more likely possibility. Bloodstream forms of *T. brucei* possess considerable quantities of proteolytic activity that, in the absence of exogenously added inhibitors, may degrade any proteolytically sensitive proteins (Lonsdale-Eccles and Mpimbaza, 1986; Lonsdale-Eccles and Grab, 1987; Lonsdale-Eccles, 1991).

In order to further analyse the activating effect of PA700 on the MCP-Tb, the trypsin-like, chymotrypsin-like and peptidyl glutamylpeptide hydrolysing activities need to be considered separately. These activities do show some overlap, particularly with respect to their reaction with different protease inhibitors. For example, chymostatin, inhibits the chymotrypsin-like activity and to a lesser extent the trypsin-like activity of the proteasome (Russo *et al.*, 1993). Likewise, leupeptin inhibits both trypsin-like and chymotrypsin-like activities of the proteasome (Chapter 3; Lomo *et al.*, 1997). Proteasome specific inhibitors such as lactacystin and Z-Leu-Leu-Leu-H block all the peptidase activities of the proteasome (Coux *et al.*, 1996). Since it has not yet been possible to selectively inhibit each of the different proteasome activities, kinetics of hydrolysis of peptide substrates by individual activities have only been estimated based on the assumption that the substrates used are specifically hydrolysed by their respective proteasome activities. However, peptide substrates are also not absolutely specific and two or more activities may hydrolyse the same substrate. It is against this background that the results of the effect of PA700 on the kinetic parameters of the hydrolysis of peptide substrates must be assessed. PA700 has the general effect of increasing the V_{max} and decreasing the apparent K_m for each of the three substrates tested (Table 6.1). Thus, it seems likely that the effect of PA700 is more complex than a simple alteration of access to the MCP-Tb active sites.

Because the phosphatase activity of PA700 seems to be essential for the enhancement of the protease activity of the trypanosomal proteasome, it seems likely that there is interaction between these different components. Although an equivalent to PA700 has not yet been found in *T. b. brucei* itself, the results presented here demonstrate that trypanosomal MCP-Tb

activity may be subject to similar regulatory mechanisms as mammalian proteasomes. The results also imply conserved functionality between the proteasomes of trypanosomes and their mammalian hosts, even though the organisms have remote phylogenetic relationships.

Chapter 7

General discussion

Trypanosoma brucei is widespread in Africa. It causes considerable losses in productivity in domestic animals and the subspecies *T. b. rhodesiense* and *T b. gambiense* are responsible for life-threatening diseases in man (WHO, 1986; Kuzoe, 1993). Currently, control of trypanosomiasis in domestic livestock involves eradication of the insect vector, the tsetse fly (*Glossina sp.*), the exploitation of trypanolerant livestock, and administration of trypanocidal compounds. Chemotherapy and chemoprophylactic agents are widely used across the African continent (Wang, 1995). While all the compounds used in domestic livestock have been on the market for over 30 years, very little is known about their modes of action. [Eflornithine (DFMO) is the only trypanocide whose mode of action is clearly known—it is an inhibitor of polyamine synthesis (Fairlamb and Cerami, 1992)]. Unfortunately, the efficiency of many of these compounds is being impeded by the emergence of drug resistant trypanosomes (Leaches and Roberts, 1981) and very little is known about the molecular basis of this resistance. In addition, vaccine development has been impeded by the inherent ability of trypanosomes to undergo antigenic variation—the primary mechanism for evasion of the host's immune responses (Cross, 1990). An understanding of the unique biochemical and molecular features of trypanosomes may allow better use of current trypanocides, may lead to a more informed approach to the control measures which should be adopted under specific circumstances, and may aid in the development of vaccines or chemotherapeutic agents.

The present study focussed on a high molecular mass multicatalytic protease (MCP-Tb) from *T. brucei*. MCPs (also called proteasomes) have been studied in eukaryotic and archaebacterial organisms (Coux *et al.*, 1996; Tanaka, 1998) and the 3-dimensional structure of MCP from *Thermoplasma* has been determined (Löwe *et al.*, 1995). The 20S proteasome is a cylindrical particle that contains the active site threonine residue located inside the cylinder (Seemuller *et al.*, 1995). Access to the interior seems to be regulated by other large (11S and 19S) protein complexes (Coux *et al.*, 1996; Tanaka, 1998) that appear to cap the ends of the cylinders. Association of the proteasome with either of these caps greatly enhances the hydrolysis of fluorogenic peptides (Chu-Ping *et al.*, 1992; Chu-Ping *et al.*, 1994; DeMartino *et*

al., 1996). Whereas complexes of the 20S proteasome with the 11S particle (also called PA28) can only degrade peptides and do not require ATP for activation (Chu-Ping *et al.*, 1992), complexes of the 20S proteasome with the 19S particle (also called PA700) degrade both peptides and proteins, particularly ubiquitinated proteins, and require ATP for activation (Coux *et al.*, 1996). Among the Trypanosomatid group of parasitic protozoa, MCP has been described in *T. cruzi* (Gonzalez *et al.*, 1996) and *T. brucei* (Lomo *et al.*, 1997; To and Wang, 1997). Based on its involvement in physiologically important processes in different cell systems (Tanaka, 1998), MCP appears to be potentially relevant to several aspects of the host-parasite relationship.

A new purification protocol was developed and used to purify MCP-Tb from bloodstream forms of *T. brucei*. Studies on substrate specificity revealed that MCP-Tb has preferences for peptides with bulky hydrophobic (Leu) or aromatic (Phe, Tyr) residues at P_1 as well as polar residues with either basic (Arg) or acidic (Glu) residues at the same subsite. This is similar to MCP-rbc, the proteasome from red blood cells. The identity of MCP-Tb as a proteasome was further demonstrated by its susceptibility to a range of inhibitors. The trypsin-like activity of MCP-Tb was insensitive to the cysteine and serine protease inhibitor N-Tosyl-Lys-CH_2Cl or the typical cysteine protease inhibitor E-64 (Barret *et al.*, 1986), but was readily inhibited by leupeptin. Similarly, the chymotrypsin-like activity of MCP-Tb was insensitive to the classical chymotrypsin-like protease inhibitor N-Tosyl-Phe-CH_2Cl but readily inhibited by chymostatin. The peptide aldehyde Z-Leu-Leu-Leu-H readily inhibited all activities of MCP-Tb. These inhibitor profiles, shared by all other proteasomes examined to date, identify MCP-Tb as a proteasome and also suggest a common evolutionary origin of the proteasomes.

MCP-Tb has caseinolytic (proteinase) activity. Indeed, it degrades β-casein at a faster rate than MCP-rbc. The high rate of casein degradation may be an adaptation for high protein turnover in the trypanosomes necessitated by rapid parasite multiplication and differentiation. Indeed, a proteasome specific inhibitor, lactacystin, prevents the intracellular stage-specific transformation of *T. cruzi* trypomastigote (non replicative bloodstream forms) into amastigote (obligate intracellular replicative forms) (Gonzalez *et al.*, 1996). Similar inhibition also blocks cell cycle progression in bloodstream and procyclic forms of *T. brucei* (Mutomba *et al.*,

1997). Thus, proteasome-mediated proteolysis appears to be essential for the transformation and proliferation of trypanosomes.

Although MCP-Tb and MCP-rbc share important structural features at electron microscopy level, they also show some significant differences that may represent the products of divergent evolution. High-resolution transmission electron microscopy showed that individual MCP-Tb particles have a four-fold symmetry. They have a diameter of about 10 nm and a dark staining center (the channel) measuring 2.8 nm in diameter. The length of MCP-Tb is about 14.5 nm long. Thus, the 3-dimensional structures of individual subunits of MCP-Tb are similar to bovine MCP-rbc. However, MCP-Tb particle preparations also exhibit peculiar end-on associations in the form of long ribbon-like chains involving ten or more units. This has not been seen before in proteasomes and its physiological significance is unclear at this time.

The relative molecular mass of MCP-Tb is 590 kDa whereas MCP-rbc is 650 kDa—the basis of this difference may involve differential glycosylation (Schliephacke *et al.*, 1991) or other post-translational modifications (Glynne *et al.*, 1993). The subunit proteins of each particle were in the M_r range 18-27 kDa. However, clear differences were observed upon resolution of these components on 2D-PAGE gels. MCP-rbc has 28 different components whereas MCP-Tb has 12 different polypeptide components. The N-terminal sequence of an MCP-Tb subunit has no obvious sequence similarities with MCP subunits from other species. Although, it is possible that regions of higher conserved sequences may be found upon obtaining a complete sequence of the MCP-Tb subunits, this amino acid sequence difference indicates that the differences in apparent M_r between MCP-Tb and MCP-rbc can probably be explained by different protein compositions.

Peptide hydrolysis by MCP-Tb was stimulated in an ATP-dependent manner by the presence of PA700. This is in agreement with earlier reports on the activation of other proteasomes by PA700 from bovine and rabbit erythrocytes (Chu-Ping *et al.*, 1994; Dahlmann *et al.*, 1995). PA700 itself has no protease activity but it does have *p*-nitrophenyl phosphatase activity that is stimulated by potassium, unaffected by ouabain, and inhibited by vanadate. PA700 elicits its ATP-dependent stimulatory activity by associating with the terminal rings of the 20S proteasome (Coux *et al.*, 1996). In the case of MCP-Tb, PA700 enhanced the various

proteolytic activities by increasing the second order rate constants of the hydrolysis of Z-Gly-Gly-Arg-AMC was 5.8 fold, of Suc-Leu-Leu-Val-Tyr-AMC 4.5 fold, and Z-Leu-Leu-Glu-βNA 6.0 fold. These values are much less than those reported in other system where values as great as 100-fold have been observed (Chu-Ping *et al.*, 1994). These reduced levels of activation observed in the present study may be explained by quarternary structures of the molecules. Electron micrographic analysis of MCP-Tb shows that there is an average of 9 individual MCP-Tb units for each chain of MCP-Tb. If PA700 can only bind to a free, open-end of a unit, then the reduced level of PA700 activation of MCP-Tb might well be accounted for by the reduced number of open ends in relation to the total number of catalytic units of MCP-Tb.

Chicken anti-MCP-Tb IgY recognizes a 590-kDa MCP-Tb on a blot. It also recognises a 27-kDa component of MCP-Tb. Lack of recognition of the other MCP-Tb components probably results from epitopes of MCP-Tb being hidden from the chicken's immune system. It is shown in the present study that anti-MCP-Tb antibodies have an inhibitory effect on the MCP-Tb protease activity (see also Lomo *et al.*, 1997). Whether MCP-Tb plays a role in the pathology of trypanosomiasis is not known. However, it is difficult to envisage the use of inhibitory antibodies to MCP-Tb to offer immuno-protection because MCP-Tb is an intracellular protease and as such it is unlikely to be accessible to anti-MCP-Tb antibodies. However, specificity of anti-MCP-Tb to MCP-Tb may be exploited to further the studies of MCP-Tb (e.g. in structural studies to determine epitope localisation by immunoelectron microscopy).

Proteasomes have previously been purified from various eukaryotic cells. They have been localised in the cytoplasm, in association with the endoplasmic reticulum, and in the nucleus (Akhayat *et al.*, 1987; Tanaka *et al.*, 1989; Peters *et al.*, 1994). The present study, however, constitutes the first report on the subcellular localisation of proteasomes from a protozoan parasite. The localisation was based on biochemical as well as immunocytochemical evidence. The distribution of MCP-Tb activity in the subcellular fractions clearly indicate that MCP-Tb is most abundant in the cytosol (75%) although some activity is also present in the nucleus (22%). It is not associated with the mitochondria, glycosomes, lysosome, nor the Golgi apparatus in the parasite.

To complement subcellular fractionation studies, immunocytochemical studies were carried out. These studies also showed the presence of MCP-Tb in the nuclei and the cytoplasm of *T. brucei*. This agrees with previous reports showing the presence of other MCPs in nuclei (Knetch *et al.*, 1991; Rivett *et al.*, 1992; Palmer *et al.*, 1996) but contrasts with reports showing the presence of MCP in the cytoplasm only and not in the nuclei (Akhayat *et al.*, 1987; Haass *et al.*, 1989; Kamakura *et al.*, 1988; Tanaka *et al.*, 1989).

The present study has unraveled some features of the trypanosomal MCP-Tb but at the same time new questions have arisen. For instance, a computer-assisted database search showed that the N-terminal sequence of a β-type subunit of MCP-Tb does not share an obvious sequence homology with subunits of other proteasomes from different cells. Such lack of sequence similarities suggests evolutionary divergence between two functionally related enzymes. It is envisaged that the protein sequence data of MCP-Tb obtained will be of use in the design of probes for molecular clone generation. Specific regions of the protein sequence may be selected for the design of probes such as PCR primers and oligonucleotides. These probes may be used to screen cDNA or genomic libraries. Recombinant proteins may be sequenced to verify the authenticity of putative positive clones, monitor the genetic stability of the clone and the proper processing of recombinant protein products.

Also, recent studies have implicated the proteasome-ubiquitin system in the endocytosis of several eukaryotic plasma proteins (Hicke, 1997 and Strous and Govers, 1999). Endocytosis is an important physiological event through which materials are internalized and degraded, and in trypanosomes, includes the degradation of anti-VSG antibodies (Webster and Grab, 1988; Webster and Fish, 1989; Russo *et al.*, 1993). It would be interesting to investigate a possible direct or indirect role of MCP-Tb in endocytosis of proteins and peptides by the parasite. Such a study may involve alternate use of different class-specific protease inhibitors and proteasome-specific inhibitors (e.g. lactacystin) on live parasites. Such studies may be extended to unravel the function(s) of MCP-Tb within the parasite, the importance of MCP-Tb in parasite survival, and the potential of MCP-Tb for drug development.

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Appendix 1

Triple and single-letter amino acid codes

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Homophenylalanine	hPhe	hF
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Pyroglutamic Acid	pGlu	pG
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix 2

Publication

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Characterization of a multicatalytic proteinase complex (20S proteasome) from *Trypanosoma brucei brucei*

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Abstract

African trypanosomes are tsetse-transmitted protozoan parasites that cause sleeping sickness in humans and 'Nagana' in animals. A high relative molecular mass multicatalytic proteinase complex (MCP) was purified and biochemically characterized from the cytosolic fraction of *Trypanosoma brucei brucei*. The isolation procedure consisted of fractionation of the lysate by high speed centrifugation, chromatography on Q-sepharose, molecular sieve filtration on Sephacryl S-300, chromatography on HA-Ultrogel and glycerol density gradient centrifugation (10–40%). The final enzyme preparation yielded a single protein band corresponding to a relative molecular mass of 630 kDa on a non-denaturing polyacrylamide gel. The enzyme hydrolyses a wide range of peptide substrates characteristic of chymotrypsin-like, trypsin-like, peptidylglutamylpeptide-hydrolysing activities determined by fluorogenic peptides, Z-Gly-Gly-Leu-NHMec, Z-Arg-Arg-NHMec and Z-Leu-Leu-Glu-βNA, respectively. The enzyme was found to have a wide variation in pH optimal activity profile, with optimum activity against Z-Gly-Gly-Leu-NHMec at 7.8, Z-Arg-Arg-NHMec at pH 10.5 and Z-Leu-Leu-Glu-βNA at pH 8.0, showing that the different activities are distinct. The enzyme hydrolysed oxidized proteins. In addition, the chymotryptic and trypsin-like activities were susceptible to inhibition by peptide aldehyde inhibitors with variable inhibition effects. The study demonstrates the presence of a non-lysosomal proteasome pathway of intracellular protein degradation in the bloodstream form of *T. b. brucei*. Further, the ability of the enzyme to hydrolyse most oxidized proteins, and the high immunogenicity exhibited suggests a possible involvement of the enzyme in pathogenesis of the disease.

Keywords: Trypanosomiasis; Multicatalytic proteinase; Antigenicity; *Trypanosoma brucei brucei*

1. Introduction

Trypanosoma brucei brucei is the flagellated protozoan parasite that is the aetiological agent of African trypanosomiasis in livestock, whose debilitating chronic course is characterized by anaemia and severe endocrinal defects (Lomo et al., 1995). Proteolysis may have a number of functions in the parasite including processing of trypanosomal proteins, degradation of proteins involved in the morphological changes occurring during their complex life cycle and the hydrolysis of host proteins for nutritional and invasion purposes. Thus, the proteinases of parasitic protozoa are of interest for several reasons, including their role in pathogenesis of parasitic diseases such as trypanosomiasis.

Abbreviations: NA, 2-naphthylamide; NHMec, 7-amino-4-methylcoumarin; DTT, dithiothreitol; E-64, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane; IgY, immunoglobulin Y; Suc, succinyl; Z, benzyloxycarbonyl

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The proteasome (EC 3.4.99.46), also known as the multicatalytic proteinase complex (MCP), is an extralysosomal high relative molecular mass proteinase found in eukaryotic cells (Kania et al., 1996). Proteasomes have also been detected in archaebacterium (Seemuller et al., 1995), yeast (Hilt and Wolf, 1992), and trypanosome (Hua et al., 1996), however, their physiological significance in these microorganisms is still unclear. In eukaryotic cells, the proteasome is thought to be the principal catalyst for protein degradation within the cytosol. Some of the suggested functions of the proteasome encompass processing of antigens (Rock et al., 1994), transcription factors (Palombella et al., 1994), regulation of cell cycle development through degradation of cyclins (Glotzer et al., 1991), degrading ubiquitin-protein conjugates (Hershko and Ciechanover, 1992) as well as breakdown of short-lived and long-lived cellular proteins (Rock et al., 1994; Hayashi and Murakami, 1995).

The present study was designed to purify and characterise the non-lysosomal MCP from *T. b. brucei* and attempt to determine its possible role in host–parasite interaction.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study were purchased from Sigma, BDH, Merck or Boehringer Mannheim and were of analytical grade or better. DEAE-cellulose (DE-53) was from Whatman Biochemical Systems, Maidstone, U.K. Sephacryl S-300, molecular weight markers, and Percoll were from Pharmacia. HA-Ultrogel, Q-sepharose, protease inhibitors, Leu-Leu-Glu-βNA and hypoxanthine were purchased from Sigma Chemicals Co., St. Louis, Mo. All other peptide substrates used were products of Bachem Feinchemikalien, Bubendorf, Switzerland. Horseradish peroxidase (HRPO) was from Seravac. Freund's complete and incomplete adjuvants were purchased from Gibco Laboratories, Life Technologies, Ohio, USA. 2,2 Azino-di-[3-ethylbenzthiazoline sulphonate (6)] ABTS was from Boehringer Mannheim, SA.

2.2. Parasite isolation

Trypanosoma brucei brucei ILTat 1.1 derived from stock EATRO 795 were used in this study. Bloodstream forms were grown in 4-month-old Sprague Dawley rats.

Trypanosome-containing blood was collected at peak parasitaemia (approximately 10^8 trypanosomes/ml) by cardiac puncture into 0.5% (w/v) citrate. The trypanosomes were separated from blood cells by a combination of centrifugation on isopycnic Percoll gradients (Grab and Bwayo, 1982) and chromatography on DEAE-cellulose (DE-53) using isotonic phosphate/saline/glucose (PSG) buffer, pH 8.0, prepared as described by Lanham and Godfrey (1970), containing 0.1 mM hypoxanthine (Lonsdale-Eccles and Grab, 1987). The trypanosomes recovered from the column effluent were washed three times in ice-cold PSG buffer, pH 7.4 containing 0.1 mM hypoxanthine and centrifuged at $1000 \times g$ for 15 min at 4°C. The pelleted cells could be frozen for subsequent enzyme purification with no appreciable loss of activity and were stored at −80°C.

2.3. Enzyme purification

All experimental procedures were carried out at 4°C. The cells (approximately 10^{10} trypanosomes) were suspended in 10 mM Tris–HCl, pH 7.0 containing 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP and ruptured in a French pressure cell (Aminco, Silver Springs) at approximately 2,500 Psi. The cell lysate was then centrifuged at $100{,}000 \times g$ for 1 h in an angle-fixed 75 Ti rotor. The pellet was washed and glycerol added to 20% (v/v) of the supernatant. The supernatant was subsequently loaded onto anion exchange column Q-sepharose (2.5 × 10 cm) equilibrated with 5 bed volumes of 10 mM Tris–HCl buffer pH 7.0 containing 0.1 mM EDTA, 1 mM DTT, 25 mM KCl, 1.1 mM $MgCl_2$, 20% glycerol and 0.02% NaN_3 (standard buffer). After removal of unbound proteins by washing with equilibrating buffer containing 100 mM KCl, bound proteins were eluted with 6 bed volumes linear gradient of 100–800 mM KCl in the same buffer. The eluted activity was pooled and concentrated (5–10 fold) by dialysing (using M_r 12,000 cut-off dialysing tubing) against

dry poly (ethylene glycol) 20,000. The buffer system was essentially as described by Dahlmann et al. (1996). The concentrated active fraction from Q-sepharose column was applied to a Sephacryl S-300 (2.5 × 96 cm) in the standard buffer. In this step, low molecular mass contaminants were removed from the proteinase. The enzyme eluted as a single peak of Z-Gly-Gly-Leu-NHMec activity which falls within the fractionation range of the bead. The active fraction from Sephacryl S-300 was further fractionated on HA-Ultrogel column of bed volume 12 ml that had previously been equilibrated with 10 mM potassium phosphate buffer, pH 6.8 containing 1 mM DTT and 20% glycerol. The MCP associated tightly with the hydroxylapatite resin. The column was washed with 60 ml of the equilibrating buffer, then the adsorbed material was eluted with 150 ml of a linear gradient of 10–500 mM phosphate in the same buffer. The enzyme sample was diluted to 5% glycerol then (1–5 mg protein) layered onto a linear gradient of 10–40% (v/v) glycerol in the standard buffer. After centrifuging for 22 h at 25,000 rpm in an SW 40 rotor at 4°C, the gradient was fractionated into 1 ml aliquots from the top. The gradient-purified enzyme was finally concentrated by dialysing against PEG 20,000 using a dialysing membrane of cut-off limit 12,000. The glycerol concentration was then brought to 20% and the purified enzyme stored at −80°C.

2.4. Assay of protease and caseinolytic activity

The reaction mixture consisted of 50 μl of 60 mM Tris–HCl buffer, 10 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT, pH 8.0, 25 μl of 200 mM Z-Gly-Gly-Leu-NHMec or other peptide-NHMec substrate and 1–5 μg enzyme protein in a total volume of 100 μl of the reaction mixture. Hydrolytic rates were measured at 37°C by continuously monitoring increases in fluorescence (excitation 360 nm; emission 460 nm) on a spectrofluorometer (7620 Microplate Fluorimeter, Cambridge Technology, Inc.) connected to an IBM compatible PC model MZ4671. The peptidylglutamyl-hydrolase activity was measured according to Mykles and Haire (1991). After incubation for 1 h at 37°C, reaction mixtures (200 μl) containing about 5 μg enzyme, 0.2 mM Z-Leu-Leu-Glu-βNA, 20 mM Hepes-NaOH (pH 8.0) were combined with 0.3 ml 1% (w/v) SDS and 1 ml borate (pH 9.0). The amount of substrate hydrolyzed was measured with a Hitachi F-200 Fluorescence Spectrophotometer (excitation 336 nm; emission 410 nm). Various inhibitors were added to the assay mixtures at desired concentrations when required and incubated for at least 30 min before the residual activities were determined. All assays were inspected for reaction linearity and enzyme concentration were adjusted to allow measurement of initial velocities only. The changes in fluoresence intensity were converted to micromoles of NHMec or βNA by determining, under the same conditions, the fluoresence intensity of a standard solution of NHMec or βNA.

The degration of dephosphorylated β-casein was determined by a gel electrophoretic method (Yu et al., 1991). Briefly, 70 μg of β-casein were incubated at 37°C with approximately 3 μg of purified enzyme in a total volume of 175 μl of 50 mM Tris–HCl, pH 8.0. 75 μl aliquots of the reaction mixture were subjected to SDS-PAGE and the gels stained with Coomassie Blue. After destaining with a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid, the band corresponding to the dephosphorylated β-casein were excised with a razor blade transferred to small test tubes and eluted by shaking overnight with 1.5 ml of 25% pyridine at room temperature. Blanks were obtained by excising a band of the same size from the background surface of each gel and subjecting it to the same treatment. As controls, 75 μl aliquots of each reaction mixture at time 0 were run in every gel. The absorbance were measured spectrophotometrically at 605 nm.

2.5. Polyacrylamide gel electrophoresis

Electrophoresis under non-denaturing conditions was performed on polyacrylamide (3–10% gradient) gels whereas the SDS-PAGE was conducted on 5% stacking and 12.5% separating gels as described by Laemmli (1970). Proteins were detected by either Coomassie Brilliant Blue. Protein estimation were done by the method of Bradford (1976) using ovalbumin as the standard. The chromatographic elution was monitored at 280 nm.

2.6. Antibodies

Polyclonal antibodies were raised in chickens against the purified enzyme. Briefly, 50 μg of the purified enzyme was emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly into the breast muscle of chickens. Subsequently, three doses of 50 μg antigen were given 2, 4 and 6 weeks after the initial injection, in incomplete Freund's adjuvant. The IgY antibodies were then isolated from the chicken egg yolk as described by Polson et al. (1985). A preimmunization sample was obtained from the yolk of eggs laid by the same chickens prior to immunization.

The reactivity of the antibody against the purified MCP was tested by an enzyme-linked immunosorbent assay procedure. Briefly, the purified enzyme was immobilized in 96-well polyvinyl chloride microtiter plates (0.1 μg in 100 μl of phosphate buffered saline, pH 7.2) by an overnight incubation at 4°C. After washing, 200 μl of 0.5% BSA-PBS was added to each well (incubated for 1 h at 37°C) to reduce non-specific binding of antibody. The plates were washed again in PBS-Tween (0.1% (v/v) Tween 20 in PBS) and drained. Serial two-fold dilutions, starting from 1 mg/ml IgY, was prepared on the plate in 0.5% BSA-PBS and incubated (100 μl, 2 h at 37°C). The plates were washed three times with PBS-Tween and further incubated for 1 h at 37°C with 100 μl of rabbit anti-chicken IgY conjugated to horseradish peroxidase (HRPO) diluted 1:300 in 0.5% BSA-PBS. Following washing, the peroxidase activity was measured with 100 μl of substrate solution (7.5 mg ABTS (chromogen), 7.5 μl H_2O_2 in 15 ml of 0.15 M citrate phosphate buffer, pH 5.0) added to each well. The colour was

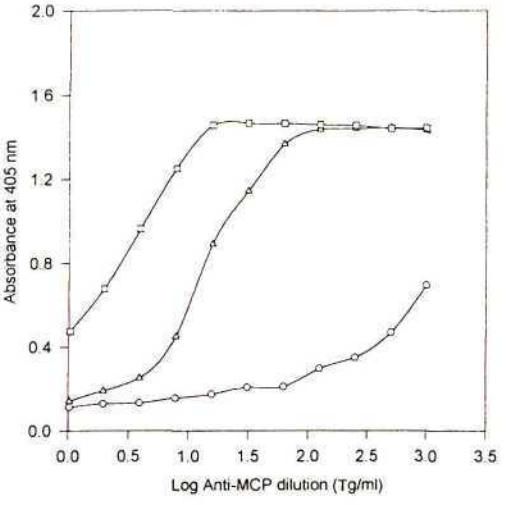

Fig. 2. Determination of antibody titre by enzyme-linked immunosorbent assay (ELISA). Purified antigens were adsorbed onto a microtitre plate then incubated with a pre-immune sample (– ∘ –) antibody at week 3 (– △ –) and week 6 (– □ –) of immunization. The bound antibody was detected by HRPO-rabbit anti-IgY using appropriate substrate.

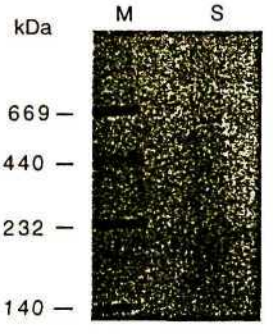

Fig. 1. Non-denaturing polyacrylamide (3–10% gradient) gel electrophoresis of the purified MCP. The gels were stained with Coomassie Brilliant Blue. The M_r markers used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and LDH (140 kDa).

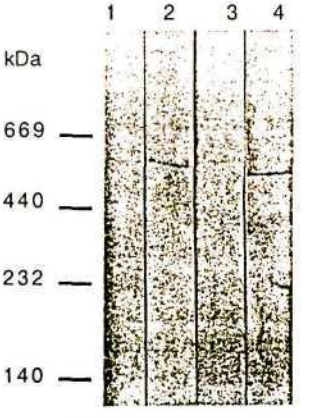

Fig. 3. Immunoblot analysis of purified multicatalytic proteinase. Lane 1: Immunoblot analysis with pre-immune sample; lane 2: incubation with chicken anti-MCP antibody; lane 3: analysis with chicken anti-variant surface glycoprotein antibody; lane 4: immunoblot analysis with anti-MCP IgY of crude trypanosome lysate.

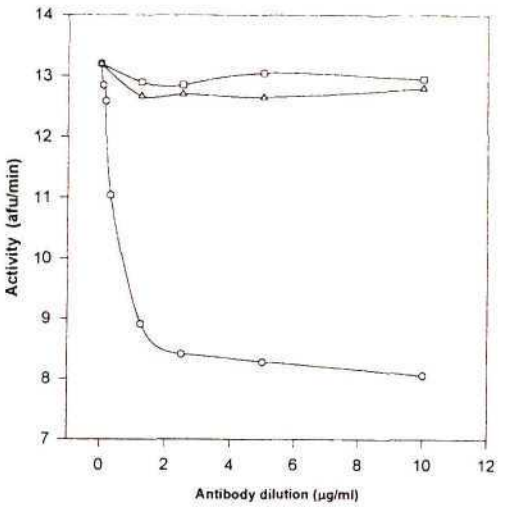

Fig. 4. Effect of anti-MCP antibody on the purified enzyme activity. The enzyme was incubated for 30 min with various dilutions of the IgY preparation and the residual activity against Z-Gly-Gly-Leu-NHMec was determined as outlined in Section 2. The activity was expressed as arbitrary fluoresence units per min.

allowed to develop against the background of controls. The enzyme reaction was stopped by addition of 50 μl of 0.1% (m/v) NaN_3 in citrate-phosphate. Absorbance of each well was read at 405 nm on a Titertek ELISA plate reader. The specificity of the IgY preparation was assessed by Western blot analysis (Towbin et al., 1979).

To assess the effect of the anti-MCP antibody on the activity of the purified enzyme, 20 μl of the enzyme was incubated for 30 min with various dilutions of the IgY preparation. Residual activity was then measured using 50 μM Z-Gly-Gly-Leu-NHMec at 37°C (Fig. 4).

3. Results

3.1. Purification

The steps used for isolating multicatalytic proteinase (MCP) from *Trypanosoma brucei* are summarized in Table 1. The MCP was purified over 60-fold with an overall recovery of 7%. The Q-sepharose chromatography of the high speed supernatant fraction resulted in a single peak of chymotryptic activity. The total recovery of the MCP chymotrypsin-like activity from the Q-sepharose column was about 20%. It should be noted that this peak degraded several oligopeptide substrates including Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, Z-Gly-Gly-Leu-NHMec and Z-Leu-Leu-Glu-βNA known to be respective substrates for cysteine protease, trypsin-like, chymotrypsin-like and peptidylglutamylpeptide hydrolysing activities, albeit with non-absolute specificities.

Further purification was achieved by molecular exclusion chromatography on sephacryl S-300. Molecular-sieving analysis of the native enzyme on the same column indicated a high relative molecular mass protein of 650 kDa. The gel filtration column effectively eliminated low molecular mass contaminants and perhaps higher M_r proteins eluting in the void volume. The active fractions bound strongly to the HA-Ultrogel and elute as a sharp peak of activity that coincided with a small protein peak. The total recovery from the HA-Ultrogel column was about 10% with about 31-fold purification. The final purification was achieved by glycerol density gradient (10–40%) sedimentation.

Table 1
Purification scheme for trypanosomal multicatalytic proteinase. All activities were determined using substrate Z-Gly-Gly-Leu-NHMec at a final concentration of 50 μM. Data presented are mean values from three separate preparations starting from 5×10^9 parasites

Fraction	Total protein (mg)	Total activity (μmol · min^{-1})	Specific activity (μmol · min^{-1} · mg^{-1})	Purification (-fold)	Yield (%)
Lysate	279.000	34.20	0.1226	1	100
HSS	203.000	14.00	0.0690	[a]	41
Q-Sepharose	8.070	6.970	0.8640	7	20
Sephacryl S-300	1.920	6.130	3.1930	26	18
HA-Ultrogel	0.874	3.304	3.7800	31	10
Dens. grad. sed	0.308	2.313	7.5100	61	7

[a] Specific activity decreased; much of the activity remained in the pellet.

Table 2
Substrate specificity of purified MCP. The enzyme activity was determined at 37°C in 30 mM Tris/HCl, pH 8.0, containing 10 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT with various substrates at a concentration of 25 μM

Substrate	Enzyme activity ($nmol \cdot min^{-1} \cdot ml^{-1}$)
Gly-Ala-Met-NHMec	3.2
Z-Gly-Gly-Leu-NHMec	29.1
Suc-Leu-Tyr-NHMec	7.7
Ac-Ala-Ala-Tyr-Mec-NHMec	13.2
H-Ala-Ala-Phe-NHMec	15.5
Suc-Ala-Ala-Phe-NHMec	5.7
Glu-Gly-Gly-Phe-NHMec	0.0
Z-Arg-Arg-NHMec	9.1
Z-Phe-Arg-NHMec	18.9
Pro-Phe-Arg-NHMec	20.6

The purity of the final preparation was assessed by using polyacrylamide gel electrophoresis (Fig. 1). The enzyme migrated as a single band of about 630 kDa on a non-denaturing (3–10%) gradient gel. The M_r obtained was slightly less than that for similar enzyme complexes reported in archaebacterium and yeast cells (Hilt and Wolf, 1992; Seemuller et al., 1995).

3.2. pH stability and optimum pH

Purified MCP was preincubated at various pH at room temperature for 30 min. The remaining proteinase activity was then determined with Z-Gly-Gly-Leu-NHMec at pH 8.0. The enzyme was stable at between pH 4.0 and 11. The pH optimum for hydrolysis of Z-Gly-Gly-Leu-NHMec in a four system buffer designated AMT (acetate, Tris, MES and Na_2EDTA) was 7.8, Z-Arg-Arg-NHMec pH 10.5, Z-Leu-Leu-Glu-βNA pH 8.0. That optimum activities are distinct points to the fact that these are different activities on the same enzyme (multicatalytic) rather than the same activity hydrolyzing different substrates in a non-specific manner.

The activities of the purified MCP on various other synthetic substrates are shown in Table 2. The enzyme hydrolysed substrates with an arginine residue at the P1 position which in general are good substrates for trypsin-like proteinases. The MCP enzyme showed a broad specificity hydrolysing a number of synthetic substrates with a remarkable preference for aromatic or hydrophobic amino acid at P1 position. The substrates with glutaryl or succinyl blocking groups were not good substrates for MCP.

Table 3
Effect of various proteinase inhibitors on the activity of MCP. Purified enzyme was incubated for 30 min at 25°C with inhibitors in assay buffer as described in Section 2. The enzyme activity was determined at 37°C against Z-Gly-Gly-Leu-HMec or Z-Arg-Arg-NHMec

Inhibitor	Concentration (μM)	Inhibition (%)	
		Z-Gly-Gly-Leu-NHMec	Z-Arg-Arg-NHMec
No addition		0	0
Chymostatin	125.0	57	58
	62.5	48	18
Leupeptin	125.0	43	97
	62.5	36	82
Tosyl-Lys-CH_2Cl	125.0	5	9
	62.5	0	4
Tosyl-Phe-CH_2Cl	125.0	11	0
	62.5	3	0
Pepstatin A	125.0	0	0
	62.5	0	0
Cystatin	125.0	0	0
	62.5	5	11
E-64	125.0	0	3

Indeed, the MCP failed to hydrolyse Glu-Gly-Gly-Phe-NHMec which is a good substrate for classical chymotrypsin-like enzymes.

3.3. Effect of protease inhibitors

Table 3 shows the effects of protease inhibitors on trypsin-like and chymotrypsin-like activities of trypanosomal MCP. The trypsin-like activity assayed with Z-Arg-Arg-NHMec as the substrate was reasonably inhibited by leupeptin and to a lesser extent by chymostatin. TLCK and TPCK had minimal effects on the trypsin-like and chymotrypsin-like activities. Also, the two activities were equally insensitive to the classical cysteine protease inhibitors such as cystatin, E-64, or antipain.

Qualititative analysis of the antibody preparation was done by an enzyme-linked immunosorbent assay method (Fig. 2). The antibody was then used to probe a Western blot of a crude lysate and purified MCP. Fig. 3, lanes 2 and 4 show the antibody binding to the antigen of size 630 kDa in the crude lysate and purified sample, respectively.

4. Discussion

In this report we demonstrate the presence of a trypanosomal proteasome and attempt to elucidate its properties in the light of its established characteristics in mammalian (Kuehn and Dahlmann, 1996), lobster (Mykles and Haire, 1991), yeast (Fujiwara et al., 1990) and archaebacteria (Seemuller et al., 1995) sources. It is noted that the trypanosomal proteasome is highly immunogenic hence may contribute significantly to the pathogenesis of the disease. Further, the ubiquitous existence of the proteasome across the prokaryotic and eukaryotic organisms no doubt confirm its pivotal role in the survival of living organisms.

The established primary function of the proteasome is to catalyze the non-lysosomal degradation of the vast majority of cell proteins (Coux et al., 1996). It also degrades short-lived enzymes such as ornithine decarboxylase (ODC), which is rate limiting in polyamine biosynthesis. Because polyamines are toxic in high levels, cells have evolved negative feedback mechanisms, which cause the inactivation and rapid degradation of ornithine decarboxylase. Accumulation of polyamines induce the specific inhibitory protein, antizyme (Hayashi and Murakami, 1995), which binds to ornithine decarboxylase, inactivates it and triggers its degradation by the 26S proteasome, provided ATP is present. Recent reports have indicated that 13 out of the 14 major subunits of the proteasome are essential for the viability of yeast cells (Fujiwara et al., 1990; Hilt and Wolf, 1992).

Although the MCP hydrolyzes a wide range of peptide substrates, its activity appears markedly affected by adjacent blocking groups of succinyl and glutaryl species at the N-terminal end of the peptides. The two blocking groups may cause steric hindrance to the S_2 and S_3 subsites in the peptide substrate binding cleft. Alternatively, the enzyme might be sensitive to the multiple negative charges on these blocking groups. Direct comparisons of this enzyme with an enzyme recently characterised by Hua et al. (1996), are difficult because the various substrates used are not the same. However, Pro-Phe-Arg-NHMec and H-Ala-Ala-Phe-NHMec are common to both studies but their relative rates of hydrolysis are considerably different. We observed a 1.5-fold difference in their rates of hydrolyses while Hua et al. (1996) observed a 6-fold difference. Hence it seems likely that the enzymes are different.

A complex group of proteins referred to as cyclins are involved in regulation of the eukaryotic cell division via their interaction with cyclin-dependent kinases (CDKs). The appearance and disappearance of particular active kinase complexes during different phases of the cell cycle is regulated by synthesis and proteolytic degradation of specific cyclins. There is evidence that proteasome might be important for cell cycle regulation by degrading cyclins that act in different phases of the cell-cycle (Glotzer et al., 1991). Disruption of either one of the two genes encoding for subunits of the proteasome has been reported to arrest the proliferation and viability of yeast cells (Fujiwara et al., 1990). Consequently, an attractive hypothesis follows that disruption of trypanosomal proteasome activity in vivo may arrest progression of the parasite multiplication and transformation. In bloodstream trypomastigotes, experiments that relate the cyclin levels with the proteasome activity in different developmental stages,

namely; the proliferative stages ('long slender') in which the parasite undergoes binary fission and non-proliferative stages ('short stumpy') in which it is incapable of division, should show whether or not the proteasome catalyzes degradation of proteins necessary for the transformation or multiplication of the parasite.

At peak parasitaemia, the antibody response results in a major trypanolytic crisis in which a whole range of biologically active factors including proteases are released into the bloodstream of the host. Immunogenic molecules such as proteasome may form an antigen–antibody complex, which in turn activates the complement system leading to damage of the host tissues (Lomo et al., 1995). Alternatively, the released trypanosome-borne proteases may accelerate the degradation of host proteins. The emaciation and muscular wasting that characterize chronic, debilitating trypanosome infection may be at least in part, due to acceleration of this degradative process in muscle in such a pathological state. This hypothesis will however be verified by immunoblot analysis of the serum samples from animals chronically infected with *T. b. brucei*. The observation that trypanosomal 20S proteasome is 630 kDa as opposed to the mammalian 20S proteasome which is 700 kDa (Coux et al., 1996) should enable us to discriminate between the host and the parasite-derived enzyme in the likely event of cross-reactivity between the two enzymes.

In conclusion, the trypanosomal proteasome is of considerable biological interest because of the myriad functions it is likely to play in the parasite and host–parasite relationships. This enzyme may be a promising target for the development of new chemotherapy to control trypanosomiasis and our generation of inhibitory antibody should prove valuable in dissecting the possible functions of the enzyme.

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