Endolysosomal proteolysis and its regulation

by

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PREFACE

The experimental work described in this thesis was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, from March 1999 to December 2001 under the supervision of Professor Clive Dennison.

These studies represent original work by the author and have not been submitted in any other form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

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ABSTRACT

The endolysosomal system is a multifunctional system and is involved in catabolism, antigen presentation and regulation of hormones. The descriptions of, and functions assigned to organelles within the system are often applied using different criteria. Further, the properties of the hydrolases within the system, and the organelles that house them are usually studied in isolation from one another. Considering that the endolysosomal system may be extremely dynamic, an understanding of the system as an integrated whole is a necessary first step. Thus, a review of the endolysosomal system was undertaken. It was determined that the enzymes within the endolysosomal system are probably subject to 'organelle-dependent' regulation, i.e. the organelles create the appropriate luminal conditions for these enzymes to work. It is also likely that the effectors of these luminal conditions are regulated in a manner that is related to GTPase networks that control much of the cell's functions. The organisation of the endolysosomal system may be hierarchical, with proteases being downstream effectors of a system that is regulated at the whole body level.

The main endoprotease class within the endolysosomal system are cysteine proteases. A literature review suggested that these enzymes may not be redox regulated within the endolysosomal system. However, the lysosomal endoprotease cathepsin B has been implicated in many pathologies where it is operating in pH and redox conditions different from the endolysosomal system. To study this, cathepsin B was isolated from bovine livers using a novel procedure that exploits the ability of tertiary butanol to temporarily inhibit protease interactions in tissue homogenates. This would prevent artefactual, as well as protease-inhibitor interactions. This novel procedure resulted in increased yields of cathepsin B. Cathepsin D, an aspartic protease, was isolated using established methods.

In order to test the hypothesis that cathepsin B may be redox regulated *in vivo*, cathepsin B activity and stability were measured in cysteine and/or cystine-containing redox buffers. Cathepsin B activity in cysteine-containing buffers was similar at pH 6.0 and pH 7.0, over all thiol concentrations tested. In contrast, the stability of the enzyme was greater at pH 6.0 than at pH 7.0. This suggests that the enzyme's operational pH *in vivo* may be < pH 7.0. The activity of the enzyme was depressed in glutathione-containing buffers. When assessed in cysteine:cystine redox buffers (pH 6.0 – 7.0) cathepsin B was active over a broad redox potential range, suggesting that cathepsin B activity may not be redox regulated.

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Originality	A+
Insight	A+
Punctuality	C
Humour	B+
Ability to learn new stuff	A+

Overall assessment: A+. Thank you for your integrity too.
"If at 20 you are not a revolutionary, there is something wrong with your heart. If at 30 you are still a revolutionary, there is something wrong with your head."

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This thesis is dedicated to my mother and grandparents. Three very special people.

LIST OF ABBREVIATIONS

AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-tris
ARF	ADP ribosylation factor
ATP	adenosine triphosphate
AV*i*	immature autophagic vesicle
AVd	mature autophagic vesicle
BLAST	basic local alignment search tool
BSA	bovine serum albumin
C	N,N'-methylenebisacrylamide
CAS	complex adaptive systems (CAS)
Cbz	carbonyloxybenzoyl
CCV	clathrin coated vesicle
cd-MPR	cation dependent-mannose 6 phosphate receptor
CHO	chinese hamster ovary
ci-MPR	cation independent-mannose 6 phosphate receptor
ddH_2O	dionised distilled water
$dist.H_2O$	distilled water
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
d(t)	delay time of inhibition
DTT	dithiothreitol
E_o	intial enzyme concentration
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino) butane
ECV	endosome carrier vesicle
EDTA	ethylene diamine tetra acetic acid
ER	endoplasmic reticulum
GAP	GTPase activating protein

GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	guanidine diphosphate
GILT	γ-interferon inducible lysosomal thiol transferase
GSH	glutathione
GSSG	oxidised glutathione
GTP	guanidine triphosphate
HRP	horseradish peroxidase
Hsc 73	constitutively expressed heat shock protein of 73 kDa
hsp	heat shock protein
i	inhibition concentration
[^{125}I]-tynSS-PDL	[^{125}I]iodotyramine linked to poly-D-lysine by a 3-(propionyldithio)propionic acid spacer
IG	immature granule
k_{cat}	catalytic constant
K_{eq}	equilibrium constant
K_i	inhibition constant
K_{ic}	competitive inhibition constant
K_{iu}	uncompetitive inhibition constant
K_m	Michaelis constant
K_{mapp}	apparent Michaelis constant
k_{obs}	observed rate constant
kat	katal
LAMPs	lysosomal associated membrane proteins
LDH	lactate dehydrogenase
mA	milliamps
MCA	metabolic control analysis
MES	2-(*N*-morpholino)ethanesulfonic acid
MPR	mannose 6-phosphate receptor

Mss4	mammalian supressor of sec 4
MVB	multi-vesicular body
NADH	beta-nicotinamide adenine dinucleotide (reduced form)
NADPH	beta nicotinamide adenine dinucleotide phosphate (reduced form)
P	product concentration
PDI	protein disulfide isomerase
RA-BSA	reduced alkylated BSA
RNAse	ribonuclease A
RS^-	thiolate form of cysteine
RSH	thiol form of cysteine
RT	room temperature
S_o	initial substrate concentration
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNARE	soluble N-ethylmaleimide sensitive factor receptor
SOC	self-organised criticality
T	acrylamide
$t_{1/2}$	half-life
t-SNARE	target SNARE
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tfn	transferrin
TGN	trans-Golgi network
THI	tetrahedral intermediate
v	initial velocity
V	limiting rate
V^{app}	apparent limiting rate
v-SNARE	vesicle SNARE
V-ATPase	vacuolar ATPase

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CHAPTER 1

INTRODUCTION

The present study of the endolysosomal system was motivated by a number of questions which had arisen in the course of previous studies in this laboratory, especially concerning the possible involvement of endolysosomal proteases in invasive cancer. In this, the enzymes are secreted from the endolysosomal system and operate extracellularly, under conditions which may be very different in terms of pH, ionic strength and redox potential. How are the enzymes secreted and could they function under extracellular conditions? Moreover, immunolabelling studies in this laboratory had shown that *ras* transformation of the MCF-10A immortal-but-not-tumourigenic human breast cancer cell line, into the tumourigenic MCF-10AneoT cell line, caused a marked change in the distribution of 'lysosomes' in the cell. How was this to be interpreted? Finally, looking ahead, many studies on the possible therapy of protease-based diseases have aimed at the rational design of specific inhibitors, targeting the protease active site, and which would thus largely be competitive inhibitors. Is this a valid approach and could it be applied to endolysosomal proteases involved in cancer and other diseases?

Early on, a schism in the literature was recognised, between groups who could broadly be called "biochemists", whose work is based largely on organelle fractionation, and "cell-biologists", whose work is largely based on microscopy, histochemistry and immunocytochemistry. The extent of this schism is illustrated by the fact that the two groups attached completely different meanings to the term 'lysosome', for example. Clearly some reconciliation between the two bodies of literature was needed. Moreover, there appeared to be a complete void in the literature concerning the effect of redox potential on endolysosomal proteases. This was surprising as cysteine proteases predominate in the endolysosomal system and these enzymes are readily inactivated by oxidation during isolation. Moreover, there exists a cysteine transporter which pumps cysteine, an effective reducing agent, into the 'lysosome': what could its purpose be?

It thus came to be realised that a necessary preface to any experimental work aimed at addressing the questions outlined above, would be a comprehensive review of the endolysosomal system and its proteases, with a view to establishing what is known about the system and how endolysosomal proteases operate, both inside and outside of the

system, and how proteolysis in the system may be regulated. In the event, this review proved to be a larger task than anticipated. The literature is vast and each paper had to be carefully interpreted, for example in terms of, "what does this author actually mean when they use the term 'lysosome'?", and many papers, in effect, needed translating from the language of biochemistry to that of cell biology and *vice versa*. Much information also needed interpretation and integration along the lines of, "how would this enzyme operate in the conditions created by this organelle system?". In this task the author was perhaps uniquely placed as this laboratory has solid foundations, and international collaborations, in protein biochemistry, enzymology and cell biology, including all levels of microscopy and immunocytochemistry. A holistic consideration of the properties of the enzymes, established *in vitro*, with their operation in the context of the endolysosomal system is thus a feature of the studies reported here.

In the end, the review of the endolysosomal system became as much a work in itself as a preface to laboratory work. For this reason, it was published as an independent study and, in the structure of this thesis, it has been given a chapter of its own (Chapter 2). Chapter 3 comprises an omnibus, "Materials and Methods" chapter. The review reported in Chapter 2 confirmed the void in information pertaining to the effect of redox potential on endolysosomal proteases and the experimental work reported in this thesis (Chapter 5) is largely aimed at filling this gap. Before the enzymes could be studied, however, they had to be isolated and the methods used, including some novel approaches developed in the course of this study, are reported in Chapter 4. In Chapter 6, a look forward is taken to consider the possibilities for controlling inappropriate proteolytic activity, in pathologies such as invasive cancer, either by the use of rationally designed protease inhibitors or by alternative strategies suggested by the insights provided by this study. Chapter 7 partly comprises a discussion in which the findings on the effects of redox potential are interpreted in the context of the endolysosomal system, reviewed earlier. Also reported in Chapter 7 is a holistic, retrospective, consideration of endolysosomal proteolysis, leading on to the development of a model describing the operation of endolysosomal proteolysis in the context of the whole body system.

CHAPTER 2

A REVIEW OF ENDOLYSOSOMAL PROTEOLYSIS AND ITS REGULATION

2.1 Introduction

The principal protein-degrading systems within the cell are the endolysosomal, proteosome-ubiquitin and calpain systems. In contrast to the ubiquitin and calpain proteolytic systems, the endolysosomal system carries out non-specific, bulk proteolysis. The endolysosomal membrane creates a sealed, limited environment that allows for optimum functioning of its hydrolases, and yet prevents inappropriate autodegradation. The membrane also houses transporters that remove amino acids generated by proteolysis to the cytoplasm.

Apart from degradation *per se*, the endolysosomal system is also involved in related functions such as regulation of signal transduction, antigen presentation, and storage. These functions are split among the various organelles making up the system, which may be thought of as distinct, but connected environments. At the most basic level the operation of the endolysosomal system of all cells types consists of enzymes that are responsible for degrading substrate that enters the endolysosomal system. The substrate, in turn, must be channelled to the enzymes. Both the enzyme and substrate are maintained in (separate) sealed compartments prior to proteolysis. These sealed environments must be capable of meeting, with creation of an environment that allows for optimal degradation of the substrate. As shown in Fig. 2.1, there exists the possibility of regulation at five points: i) at the point of acquisition of substrate by the endolysosomal system, ii) at the meeting of enzyme (E) and substrate (S) by fusion of their respective compartments, iii) at the establishment of conditions supporting proteolysis (or not) within the (fused) organelle and, possibly (iv) at the point of egress of product from the system. Point (v) represents the possibility of an enzyme being retained in a lysosome for a greater or lesser period (perhaps in an inactive form, but in any event separate from substrate). In terms of flux through the system, regulation at (i) and (ii) could be called 'upstream regulation' while regulation at (iii), (iv) and (v) could be termed 'downstream regulation'.

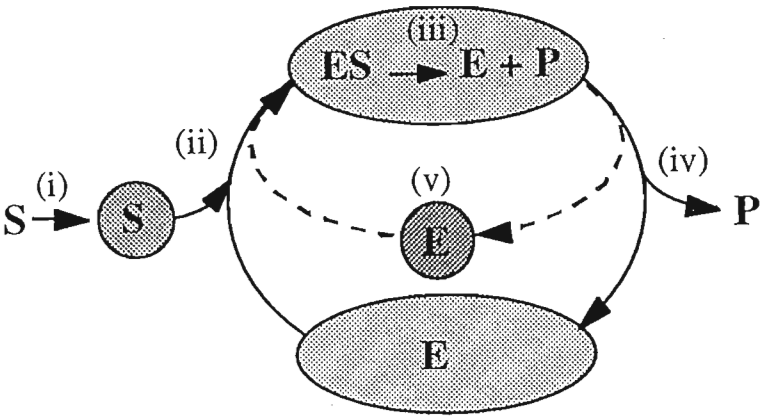

Figure 2.1. A schematic representation of the endolysosomal system, showing possible points at which regulation may be effected. The different colours indicate that the compartments shown may have different intra-luminal conditions, especially pH. (i)-(v) are possible points of regulation of the system.

Regulation of substrate acquisition into the endolysosomal system, and of organelle fusion/budding events (steps i and ii, Fig. 2.1) is effected by several signalling networks (reviewed by Guallier *et al.*, 1999; Matozaki *et al.*, 2000; Qualmann *et al.*, 2000; Sechi and Wehlan., 2000; Clague and Urbé, 2001; Di Fiore and De Camilli, 2001; May and Machesky, 2001; Stenmark and Olkkonen, 2001; Miaczynska and Zerial, 2002). The signalling networks may include small G-proteins and their signalling cascades, the actin network, and/or phosphatidylinositol 3-phosphate and associated kinases (Guallier *et al.*, 1999; Matozaki *et al.*, 2000; Qualmann *et al.*, 2000; Sechi *et al.*, 2000; Clague and Urbé, 2001; Di Fiore and De Camilli, 2001; May and Machesky, 2001; Stenmark *et al.*, 2001; Miaczynska and Zerial, 2002). These signalling networks interact with the endolysosomal system through interface molecules that are part of both endocytosis and the signalling networks (Matozaki *et al.*, 2000; Clague and Urbé, 2001; Di Fiore and De Camilli, 2001). This review will focus largely on possible regulatory mechanisms operating at points (iii) and (v), i.e. on factors which may affect the activity of the enzymes *per se*. Observations by Kominami *et al.* (1983) of the large accumulation of hepatic autophagic vacuoles containing undigested material, after treatment of rats with leupeptin *in vivo*, suggests that there may be no feedback regulation of the endolysosomal proteolysis system (e.g. from step (iv) to steps (i), (ii) or (iii), Fig. 2.1).

2.2 Organisation of the endolysosomal pathway

The basic organisation of the endolysosomal pathway in a mammalian epithelial cell is depicted in Fig. 2.2, though details may vary with cell type. Currently, there are two models to explain how the organelles within the endolysosomal pathway are related to each other (Murphy, 1991; Griffiths, 1996). While the two theories differ on the temporal organisation of the pathway, the spatial and functional aspects are very similar.

The 'maturation model' suggests that each organelle along the endocytic pathway is a transient, but distinct, compartment that matures into the next organelle along the pathway. In this model, the early endosome is envisioned as being formed *de novo* by the fusion of uncoated primary endosomes derived from the plasma membrane. This transient compartment then matures into a transient late endosome, which in turn matures into a lysosome, the terminal organelle (Murphy, 1991). Each maturation stage has its own unique set of biochemical markers associated with it. These markers and membrane components are recycled by carrier vesicles during maturation (Murphy, 1991). In a related model, proposed by Thilo *et al.* (1995), maturation occurs from the primary endosome until a pre-lysosomal compartment is formed. This compartment then communicates with the lysosome through vesicular traffic.

In the 'pre-existing compartment model', the early and late endosomes are considered to be stable, specialised compartments linked by vesicular traffic (Griffiths, 1996). The early and late endosomes are regarded as 'compartments', which are stable and do not undergo maturation, but are capable of homotypic fusion. 'Vesicles', unlike compartments, were considered incapable of homotypic fusion. Compartments are considered to be more structurally complex and to have more specialised functions compared to vesicles (Griffiths, 1996).

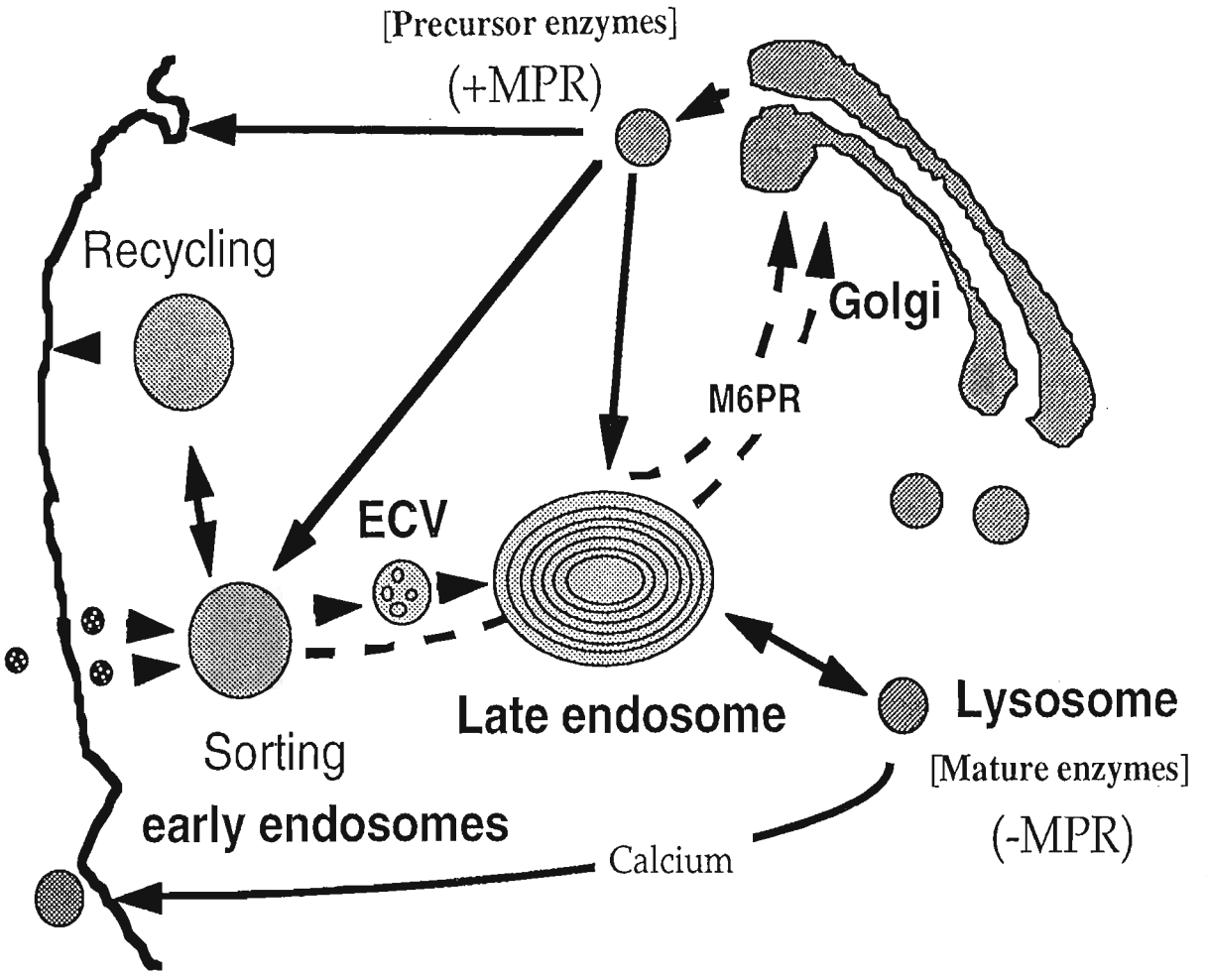

Figure 2.2. Schematic representation of the endolysosomal system. The major organelles of the endosomal system, the early endosome, late endosome and lysosome are shown. Solid arrows between these organelles may represent vesicular traffic, maturation, or direct fusion. Additional components of the endolysosomal system, i.e. phagosomes and autophagic vesicles, are not shown in this simplified diagram. .

The first organelle along the endolysosomal pathway, the early endosome (Fig. 2.2), is a major sorting station. Based on the recycling behaviour of ligand-receptor complexes, the early endosome population may be subdivided into at least two sub-populations, the sorting and recycling endosomes (Ghosh *et al.*, 1994). The luminal pH within the sorting endosome is between pH 6.3-6.5, and is generated by an H^+-ATPase and regulated electrogenically by a Na^+, K^+-ATPase (Fuchs *et al.*, 1989; Cain *et al.*, 1989). At this slightly acidic pH, ligands like low density lipoprotein (LDL) and α_2-macroglobulin (α_2M), dissociate from their cognate receptors. The receptors are recycled to the plasma membrane, whilst their ligands, LDL and α_2M, are trafficked to the late endosome (reviewed by Mellman, 1992). Some receptors, like the transferrin (Tfn) receptor can be recycled to the plasma membrane directly from the sorting endosome (fast cycle), or directed to the recycling endosome and then to the plasma membrane (slow cycle) (Sakai *et al.*, 1998). The recycling endosome may also play a role in directing receptors to the leading edge of migrating cells (Sakai *et al.*, 1998).

The next stage on the endolysosomal pathway is the endosome carrier vesicle (ECV) or multivesicular body (MVB) (Fig. 2.2). ECVs appear to be large (0.4-0.7 μm), usually spherical vesicles, often with a membrane enriched luminal content (reviewed by Griffiths, 1996; Gruenberg *et al.*, 1989; Aniento *et al.*, 1993) and are responsible for transferring material from early to late endosomes (Griffiths, 1996). Formation of ECVs is dependent on the acidification of their luminal interior and can be blocked by the vacuolar ATPase (V-ATPase) inhibitor, bafilomycin A_1 (Clague *et al.*, 1994). Indeed, the luminal pH of the ECV drops from that of the early endosome to pH 5.0-5.5 (Killisch *et al.*, 1992). Endocytosed material may not always be transferred to late endosomes via the ECV. In the mouse macrophage cell line J774, early and late endosomes are capable of fusing directly with each other (Jahraus *et al.*, 1998). This suggests that certain cell types may have specialised endocytic fusion machinery depending on their function.

Distal to the endosome carrier vesicle is the late endosome (sometimes called the pre-lysosomal compartment) followed by the lysosome. A modern definition of a lysosome is that it is, "the terminal organelle on the endocytic pathway and is devoid of recycling receptors" (Griffiths, 1996). Complicating the field of endolysosomal proteolysis is the fact that not all authors make a distinction between the late endosome and the lysosome. In the original papers of de Duve *et al.* (see de Duve, 1983), 'lysosomes' were defined on the

basis of two criteria; the existence of a limiting membrane and the presence within the organelle of acid hydrolases. Thus to many authors, lysosomes are any organelles containing acid hydrolases, i.e. 'lysosomal' enzymes[1]. However, from a modern perspective, this definition encompasses early and late endosomes, lysosomes, phagosomes and autophagosomes. Recently it has been found that not all 'lysosomal' enzymes have an acidic pH optimum (Butor *et al.*, 1995; Dehrmann *et al.*, 1996), and that not all late endosomes/lysosomes are acidic (Butor *et al.*, 1995).

Both late endosomes and lysosomes contain 'lysosomal' hydrolases and lysosome associated membrane proteins (LAMPs) (Griffiths, 1996) and both are enriched in lysobisphosphatidic acid (Clague, 1998). Lysobisphosphatidic acid is a phospholipid found in high concentrations on the internal membranes of late endosomes where it may play an important role in the degradation of glycolipids, and transport of membrane proteins and lipids (Gruenberg, 2001). Under certain conditions the two organelles may also have similar distributions in density gradients. However, there are differences, e.g. mannose 6-phosphate receptors (MPRs), and the regulatory (RII) domain of the cAMP-dependent protein kinase, are found on late endosomes but not lysosomes (Griffiths, 1996) but, to date, no specific marker has been described for lysosomes. Their ultrastructural morphologies are also different. Late endosomes have a complex morphology (Griffiths, 1996) organised by microtubules (Méresse *et al.*, 1995). In sections, they often have a multivesicular appearance, with intraluminal membrane whorls (see e.g. Tjelle *et al.*, 1996; Bright *et al.*, 1997). By contrast, lysosomes appear as roughly spherical, electron dense organelles with a simpler organisation (Griffiths, 1996; Bright *et al.*, 1997). Based on their relatively simple ultrastructure, and their markers, lysosomes may be closer to 'vesicles' than to 'compartments' (Griffiths, 1996). A significant difference between lysosomes and other 'vesicles', though, is their capacity to undergo homotypic fusion (Ward *et al.*, 1997).

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[1] In referring to 'lysosomal' hydrolases here, the term 'lysosomal' is put in quotation marks because some of these enzymes are, in fact, not limited to lysosomes but are found throughout the endolysosomal system.

Late endosomes and lysosomes are apparently in dynamic equilibrium. Fluid phase markers, like BSA-gold, are distributed between the late endosome and lysosome after extended chase periods (Griffiths, 1996). Their concentrations of LAMPs are approximately equal, suggesting that their membranes are also in equilibrium. The mechanisms involved in establishing equilibrium may include vesicular transport (Berg *et al.*, 1995), "kiss and run" events (Storrie and Desjardins, 1996) or direct fusion (reviewed by Luzio *et al.*, 2000) and may vary with cell type. Despite their close association, late endosomes and lysosomes have distinct functional differences. Although containing only 20% of the total hydrolase pool, late endosomes are the main site for proteolysis (Tjelle *et al.*, 1996; Bright *et al.*, 1997). By contrast, lysosomes contain the bulk of the 'lysosomal' hydrolase pool but only *ca.* 20% of total proteolysis takes place in lysosomes. It has consequently been suggested that lysosomes may act as storage organelles for these hydrolases (Griffiths, 1996; Tjelle *et al.*, 1996).

Fusion of late endosomes and lysosomes produces a hybrid organelle with properties of both (Luzio, 2000). Bafilomycin A1 did not decrease late endosome-lysosome fusion suggesting that acidification was not a requirement for hybrid formation (Mullock *et al.*, 1998). However, lysosome recondensation from the hybrid organelle was dependent on a functional V-ATPase (Pryor *et al.*, 2000). A further requirement for hybrid organelle formation was the presence of intra-organellar calcium and calmodulin. Intra-organellar calcium is also required for fusion and recondensation of lysosomes to and from the hybrid organelle (Pryor *et al.*, 2000).

Although the lysosome is the terminal organelle of the endolysosomal system, lysosomes should not be viewed as dead-end organelles as 'secretory lysosomes' may be a feature of many normal cells (Andrews, 2000). An increase in intracellular Ca^{2+} levels typically results in secretion of about 5-15% (rising to 60% in hematopoietic cells) of the total content of lysosomes. A rise in Ca^{2+} levels causes lysosomal membranes to fuse with the cell membrane resulting in exocytosis of the lysosomal contents (Andrews, 2000). The process is thought to be regulated by synaptotagmin VII (Martinez, *et al.*, 2000) and may constitute a mechanism for the repair of damaged plasma membrane (Reddy *et al.*, 2001). Mechanisms for the secretion of 'lysosomal' proteases are clearly of interest in the context of cancer, where these enzymes have been purported to play an extracellular role. Many cells of hematopoietic origin are capable of exocytosing multivesicular bodies (late

endosomes/lysosomes) containing internal vesicles called exosomes. These exosomes may be involved in multiple functions including T-lymphocyte stimulation (reviewed by Denzer *et al.*, 2000).

2.3 Rab proteins direct membrane fusion event within the cell.

Membrane fusion events between the various organelles within the endolysosomal system are highly regulated within a cell. As described in Fig 2.1 (step (ii)) the dynamics of these fusion events may contribute to the regulation of proteolysis within the cell. The details of these regulatory events have been reviewed extensively elsewhere (Ferro-Novick and Jahn, 1994; Schimmöller *et al.*, 1998; Valentijn and Jamieson, 1998; Gerst, 1999; Gonzalez and Scheller, 1999; Rodman and Wandinger-Ness, 2000).

In order for two membranes to fuse, at least two criteria have to be met. Firstly, the membrane fusion event must be specific. This ensures that movement along the endolysosomal pathway is vectorial. Secondly, the electrostatic force generated by two membranes in close proximity to each other must be overcome. The specificity of membrane fusion events is largely directed and regulated by Rab proteins (Schimmöller *et al.*, 1998; Rodman and Wandinger-Ness, 2000). Rab proteins belong to the Ras superfamily of small GTPases. They are highly conserved and are ubiquitously distributed throughout eukaryotic cells. Approximately 40 Rab isoforms have been described and these confer specificity to the distinct intracellular transport routes within the cell (Valentijn and Jamieson, 1998; Rodman and Wandinger-Ness, 2000).

Unique structural elements on individual Rab proteins allow them to recognise and be localised to their target membranes. Most Rabs are geranylgeranylated, enabling membrane association. However, Rab proteins will only associate with their target membranes in the GTP-bound conformations. GTP hydrolysis leads to inactivation of the Rab protein and subsequent loss of membrane association (Schimmöller *et al.*, 1998). This Rab GTP/GDP cycle is affected and regulated by several proteins such as GDP dissociation inhibitor (GDI), GTPase activating protein (GAP), mammalian suppresser of sec4 (Mss4) and GDI displacement factor (GDF) (Valentijn and Jamieson, 1998). These proteins are in turn regulated by signalling networks (Matozaki *et al.*, 2000). Thus, the fusion between substrate- and protease- containing organelles may be integrated with signalling networks.

These, networks, as will be described later, may also influence the luminal endolysosomal environment.

Rabs may play a role in the regulation of membrane budding and cargo selection into the vesicles. Rab effectors may connect a vesicle to the cytoskeleton, and thus play a role in vesicle movement. Rabs and their effectors have also been shown to interact directly with the actin network and may affect motor protein activity of vesicles on microtubules. Further, Rab effector proteins could act as molecular tethers, regulating vesicle docking with a target membrane (reviewed by Rodman and Wandinger-Ness, 2000). Recent evidence, however, suggests that Rab proteins may exert their action on the acceptor membrane (i.e. the membrane receiving the vesicle) rather than on the donor/vesicle membrane for COP-II vesicles involved in ER to Golgi transport (Cao and Barlow, 2000). Whether this is a general feature of membrane docking-fusion reactions remains to be determined.

Rab proteins may also activate the SNARE (soluble N-ethymaleimide sensitive factor receptor) fusion machinery. Thus, Rab proteins are central regulators in membrane fusion events within the cell. The SNARE fusion machinery is the mimimal requirement for fusion between two membranes (Weber *et al.*, 1998). There are two distinct groups of SNARE proteins, v-SNARES and t-SNARES, based on their membrane association. SNARE complexes are formed by the interaction of vesicle- or v-SNARES, with a cognate target- or t-SNARE on the target membrane. In order to prevent inappropriate fusion reactions, the SNARE proteins are probably protected by other factors such as Sec 1p (Schimmöller *et al.*, 1998). Deprotection of the SNARE proteins is undertaken by Rabs or Rab effectors. The cytosolic v- and t- SNARE domains are believed to associate with each other in a coiled coil structure or a closely related helical bundle structure. This association is energetically favoured and may provide some of the energy that helps drive fusion between the two membranes (Gerst, 1999). Dissociation of the SNARE complex is undertaken by NSF and ATP (Gerst, 1999).

Finally, Rab proteins also undergo an apparently futile cycle of GTP hydrolysis that is not associated with vectorial membrane fusion. This allows Rabs to act as timers for membrane fusion events, affecting the kinetics of these events, as well as the size and shape of organelles (Rybin *et al.*, 1996). This is an important property of these molecules, as it suggests a mechanism by which proteolysis could be regulated within the endolysosomal system. Rab proteins, which are localised to specific membranes, and intrinsically have the

ability to regulate rates of membrane fusion, may thus regulate the fusion events between substrate- and protease- containing organelles (step (ii), Fig. 2.1). Rab proteins, and their appropriate effectors, together with the appropriate signalling networks may play a integral role in regulating and co-ordinating proteolysis within the endolysosomal system.

2.4 Delivery of substrates into the endolysosomal system

Substrates destined for degradation can enter the endolysosomal proteolytic environment by three broad mechanisms: endocytosis, autophagy, and phagocytosis. These processes all result in environments which support proteolysis by a common set of lysosomal hydrolases. These environments are reviewed below with the aim of finding common luminal features that may provide insight into the regulation of endolysosomal proteolysis.

2.4.1 Endocytosis

Endocytosis can be divided into three distinct mechanisms: receptor mediated endocytosis, constitutive endocytosis and caveoli formation. Ligand-receptor complexes entering the endolysosomal system can be sorted into one of three pathways, i) the entire ligand-receptor complex may be recycled back to the plasma membrane, ii) the ligand-receptor complex may dissociate, with the receptor being recycled and the ligand directed further along the pathway; or iii) the entire ligand-receptor complex may be targeted to the later stages of the pathway (see Warnock, 1999 and references therein). This sorting process thus occurs within the early or late endosomes.

In contrast to receptor mediated endocytosis, substrates entering the pathway by fluid phase endocytosis are constitutively directed to further stages along the endolysosomal pathway. Pulse-chasing fluid phase markers, such as horseradish peroxidase and BSA-gold, into a cell first labels early endosomes (5 min), and with longer chase times (> 30 min) the later endosome populations become labelled (see for example Aniento *et al.*, 1993; Rabinowitz *et al.*, 1992).

2.4.2 Autophagy.

There are three distinct autophagic mechanisms: macroautophagy, microautophagy, and import of cytosolic polypeptides into the endolysosomal system. Autophagy is

responsible for the destruction of most endogenous proteins, the removal of obsolete and/or damaged organelles, cellular remodelling during differentiation, metamorphosis and ageing (Seglen and Bohley, 1992; Blommaart *et al.*, 1997). *In vitro* it may be activated by serum withdrawal or when amino acids are limiting. The regulation of autophagy has been reviewed by Blommaart *et al.* (1997).

Macroautophagy occurs when entire regions of the cytoplasm are sequestered by a membrane and degraded. This process occurs in at least three distinct stages that are characterised by morphological and biochemical changes to the sequestered cytosol. These stages from sequestration to degradation occur rapidly, with a half-life of approximately 9 min (Pfeifer *et al.*, 1978). The first stage is referred to as sequestration, and is characterised by the formation *de novo* of an organelle referred to as a nascent autophagic vesicle (AV*i*) or the phagophore. A consistent terminology to describe the various stages of autophagy has not yet emerged, and the terminology of Dunn (1990a,b) will be used here. The signals responsible for the sequestration step are not known but may involve G-proteins (Ogier-Denis *et al.*, 1995; 1996; Petiot *et al.*, 1999), or perhaps protein conjugation (see Mizushima *et al.*, 1998 and Kirisako *et al.*, 1999 and references therein).

The source of the sequestering membrane is a matter of dispute but it may originate from the rough endoplasmic reticulum (Dunn, 1990a) or the post-Golgi region (Yamamoto *et al.*, 1990a,b). AV*i*s have a distinctive structure with membrane structures and entire organelles engulfed by the forming AV*i* (see for example Fig. 1 in Lawrence and Brown, 1992). The AV*i* does not have lysosomal hydrolases or LAMPs associated with it (Dunn, 1990b; Tooze *et al.*, 1990; Rabouille *et al.*, 1993). It is therefore presumed that the AV*i* is not involved in proteolytic degradation of the engulfed cytoplasm.

The AV*i* apparently undergoes biochemical and morphological changes (maturation), acquiring lysosomal membrane proteins (like LAMPs) and hydrolases that subsequently allow it to degrade the sequestered substrate (Dunn, 1990b; Tooze *et al.*, 1990; Rabouille *et al.*, 1993). The AV*i* also develops an acidic luminal interior (Dunn, 1990b; Strømhaug and Seglen, 1993) and becomes capable of proteolysis. The fully matured vesicle is called an AVd (Dunn, 1990a,b). In contrast to the AV*i*, the AVd usually has a single membrane, the other membrane structures presumably having been degraded or recycled.

The mechanism of AVd formation from the AV*i* has not been resolved. The AV*i* may fuse directly with lysosomes, rapidly converting it to an AVd (Lawrence and Brown, 1992). Alternatively, Tooze *et al.* (1990) showed that the endocytic and autophagic pathways converge after the AV*i* and early endosome stages, respectively, resulting in formation of amphisomes. In either case, the AVd acquires its lysosomal hydrolases from the endolysosomal system.

2.4.3 Phagocytosis

Although a number of cell types are capable of phagocytosis, the most important professional phagocytes are neutrophils and mononuclear phagocytes (van Oss, 1986). This discussion will be limited to macrophages. Macrophages have a complement of cysteine-endoproteases similar to that of most epithelial cells. Presumably the components necessary for a fully functional proteolytic system are the same, or very similar, in both cell types. A notable exception is that macrophages contain the powerful cysteine endoprotease, cathepsin S (Kirschke *et al.*, 1998).

Phagocytosed particles are usually in the size range of 0.3-0.5 μM or larger. Their adhesion to the macrophage surface may depend on a number of forces: van der Waals-, hydrophobic-, electrostatic- or receptor-mediated interactions (van Oss, 1986). The adhesion of a particle to the membrane surface triggers a cascade of signalling events (reviewed by Kwiatkowska and Sobota, 1999) leading to the formation of a phagosome. Initially, the phagosome lumen resembles the extracellular environment but the phagosome soon undergoes a series of biochemical changes (maturation), accompanied by changes to the lumen environment. This process is Rab-dependent and involves fusion of the developing phagosome with endocytic organelles (Mayorga *et al.*, 1991; Pitt *et al.*, 1992; Desjardins *et al.*, 1994; Jahraus *et al.*, 1998). The changes to the phagosome include: V-ATPase-dependent acidification, and acquisition of Rabs, LAMPs and lysosomal hydrolases (Mellman *et al.*, 1986; Claus *et al.*, 1998). Some phagocytosed pathogens escape destruction by disturbing the maturation process. For example phagosomes which ingest *Mycobacterium avium* cells fail to acidify (Sturgill-Kozycki *et al.*, 1994) and those ingesting *Mycobacterium bovis* cells fail to incorporate Rab 7 (Via *et al.*, 1997) and consequently do not participate in late endocytic fusion events (Via *et al.*, 1997; Jahraus *et al.*, 1998). Thus, the pathogen escapes destruction by the hydrolases found in late endosomes/lysosomes.

The above discussions on endocytosis, autophagy, and phagocytosis are not intended to be exhaustive, but merely to describe how substrate enters the endolysosomal system, and what the requirements are for generating a proteolytic environment. The luminal requirements are summarised in Table 2.1, and are discussed in greater detail below.

Table 2.1 Common luminal features of proteolytic organelles of the endolysosomal system.

Organelle	Hydrolase	LAMP	MPR	V-ATPase	Luminal Ca^{2+}	Cysteine transporter	References
early endosome	+ cathepsin H	-	+	+	+	?	Claus *et al.* (1998); Griffiths (1996); Mellman *et al.* (1986)
late endosome	+	+	+	+	+	?	Griffiths (1996); Hasilik (1992); Mellman *et al.* (1986); Pryor *et al.* (2000)
lysosome	+	+	-	+	+	+	Griffiths (1996), Hasilik (1992); Mellman *et al.* (1986); Pryor *et al.* (2000)
hybrid organelle	+	+	+	?	+	?	Bright *et al.* (1997); Luzio *et al.* (2000); Pryor *et al.* (2000)
autophagic vacuole (AVd)	+	+	-	+	+	?	Dunn, (1990b); Lawrence and Brown, (1992); Rabouille *et al.* (1993); Tooze *et al.* (1990)
phagosome	+	+	-	+	?	?	Claus et al., (1998); Griffiths (1996); Jahraus *et al.* (1998); Mellman *et al.* (1986); Dunn, 1990b; Tooze *et al.* (1990); Rabouille *et al.* (1993)

2.5 Common features of endolysosomal lumen proteolytic environments.

2.5.1 The enzymes

Most of the soluble endolysosomal hydrolases are synthesised as preproenzymes (Hasilik, 1992). The signal peptide is cleaved co-translationally and the hydrolases fold into their precursor form within the ER lumen. These precursors undergo Asn-linked glycosylation and carbohydrate processing, which continues in the Golgi. Within the Golgi, two

enzymes, *N*-acetylglucosaminylphosphotransferase and α-*N*-acetyl-glucosaminidase, add a mannose-6-phosphate label (Kornfeld, 1986; Hasilik, 1992). The labelled precursors thus become ligands for the membrane bound MPRs which direct them to the endolysosomal system (reviewed by Kornfeld, 1986 and Hasilik, 1992). Glycosylation may also serve to protect the hydrolases themselves from destruction within the endolysosomal system (Bohley and Seglen, 1992).

There are over 50 'lysosomal' hydrolases, and some of these enzymes show no sequence homology to each other. Their recognition by the phosphotransferase therefore involves common tertiary structural features (Tikkanen *et al.*, 1997; Cuozzo *et al.*, 1998; Lukong *et al.*, 1999). There are two MPRs; the 275 kDa cation-independent MPR (ci-MPR) and the 46 kDa cation-dependent MPR (cd-MPR) (Kornfeld, 1986). These receptors have different affinities for the soluble precursors depending on their carbohydrate structure (Ludwig *et al.*, 1995). Together, these two receptors are able to efficiently sort the diverse array of soluble precursors for targeting to the endolysosomal system. Evidence also exists for MPR-independent targeting mechanisms (Hasilik, 1992). Within the endolysosomal system, proteolytic removal of a pro-piece serves to generate the mature, active enzyme.

The endolysosomal protease pool may be divided into endo- and exopeptidase pools The endoprotease pool (Table 2.2) is made up mainly of cysteine and aspartic proteases, whilst the exopeptidase pool is made up of cysteine and serine proteases. Serine proteases, which are the most numerous and diverse hydrolase class in nature, are notably absent from the endoproteolytic pool. The serine protease, cathepsin G, is found exclusively in hematopoietic cells, and is not a true endolysosomal enzyme (Table 2.2).

It is presumed that the rate-limiting steps in substrate hydrolysis are effected by endoproteases. Thus, the present discussion will focus on these enzymes, most of which belong to the C1 family of cysteine proteases (Table 2.2). The C1 family all show a common fold with the archetypal protease of this family, papain. These enzymes are bi-lobular with left (L) and right (R) domains. These domains are highly conserved and all members show common secondary structure elements in their respective domains. Indeed, differences between these proteases are usually due to deletions or insertions in the loop regions between the conserved structural elements that make up the papain fold (Kirschke *et al.*, 1998).

Table 2.2 Endoproteases found within the endolysosomal system.

Name	Catalytic group	M_r (kDa)	Operating pH	pI	Distribution
Cathepsin B	Cys	27	5-6.5	5.4	ubiquitous
Cathepsin D	Asp	42	2.8-5.0	5.5-6.5	ubiquitous
Cathepsin E	Asp	100	2.5	4.1-4.4	?
Cathepsin G	Ser	30	7.5	10	PMNLs
Cathepsin H	Cys	28	5.0-6.5	7.1	ubiquitous
Cathepsin L	Cys	29	4.5-6.0	5.8-6.1	ubiquitous
Cathepsin N	Cys	34	3.5	6.2	ubiquitous
Cathepsin S	Cys	24	5.0-7.5	6.3-6.9	?
Cathepsin T	Cys	34	6.9	?	?
Cathepsin K	Cys	27-29	6.0-6.5	?	osteoclasts
Legumain	Cys	31	4-6	?	ubiquitous

The active site is in a deep cleft between the L and R domains. The active site cysteine, Cys 25 (papain numbering), and histidine, His 159 (papain numbering), form a thiolate-imidazolium ion-pair that is responsible for catalysis. Other highly conserved residues are an asparagine (Asn 175), which is believed to orientate the imidazolium ring, and glutamine (Gln 19) which is part of the oxyanion hole. Substrate binds into the active site in an extended conformation and the carbonyl carbon of the scissile bond undergoes nucleophilic attack from the active site thiol. This results in the release of the amine product. The resultant acyl-enzyme reacts with water to release the carboxyl product (deacylation), resulting in the regeneration of the free enzyme. A detailed description of the catalytic mechanism is provided by Storer and Ménard (1994).

Why has this family of proteases been selected in preference to other proteases types? Perhaps uniquely, these enzymes have the following features that make them well-suited to the endolysosomal system:

- The nascent enzymes must be capable of being recognised by the phosphotransferase system within the ER. This recognises structural features, and not a linear sequence (Tikkanen *et al.*, 1997; Cuozzo *et al.*, 1998; Lukong *et al.*, 1999). This may thus place limits on the hydrolases that could be accommodated within the system. Gene duplication would have been a proficient mechanism for ensuring that variants of these unique enzymes would be present within the endolysosomal system.

- The lysosomal cysteine proteases are required to cleave a wide variety of substrates. Thus the active sites of these enzymes may not be optimised for a specific substrate. However, the catalytic mechanism of this class of cysteine peptidases is very efficient when compared to the catalytic mechanism employed by serine proteases, for example (Polgar *et al.*, 1986). This may off-set any loss of efficiency due to a relatively non-specific active site.

- The luminal conditions of pH and redox potential within the late endolysosomal system are designed to denature substrates, allowing for increased hydrolytic efficiency. These conditions may be in a constant state of flux, allowing many different hydrolase classes to operate within the system. The cysteine proteases must be stable to these denaturing conditions, and in addition, must be capable of operating over a broad pH range. The enzymes themselves also have to be fairly resistant to proteolysis. The ion pair of the papain superfamily appears to be active over a wide pH range (Storer and Ménard, 1994), a requirement that cannot be met by any of the other proteolytic classes. This would allow these enzymes to operate in the dynamic endolysosomal proteolytic environment (Butor *et al.*, 1995). However, in contrast to the other members of the papain superfamily the lysosomal enzymes tend to be unstable at neutral to alkaline pH values (Kirschke *et al.*, 1998). This may protect the cell against these enzymes. The enzymes themselves appear to be stable to the denaturing conditions within the endolysosomal system, and have half-lives that run from days to weeks (Bohley and Seglen, 1992).

- Some of the cysteine proteases are restricted to specific organelles. For example, cathepsin H appears to be restricted to the early endosome in J774 macrophages (Claus *et al.*, 1998). This suggests that this enzyme must have features that restrict it to a specific organelle, at least in this cell type.

- Finally, endolysosomal proteinases may be capable of regulation without the need for inhibitors. There is no evidence of intra-endolysosomal inhibitors, and yet proteolysis may be shut-down in (storage) lysosomes. This suggests that these enzymes have properties that allow them to be regulated within the endolysosomal system.

Thus, quite apart from being efficient hydrolases, these enzymes appear to fulfil a number of requirements that allow them to operate within the endolysosomal environment. Of these additional requirements, the ability to be regulated, without the need for intra-endolysosomal inhibitors, may be amongst the most significant.

Cathepsin D is an aspartic endoprotease found within the endolysosomal system. Unlike its cysteine protease counterparts, this enzyme is capable of operating at very acidic pHs. Thus cathepsin D could effect proteolysis at pHs where the cysteine proteases may be inactivated by oxidation or protonation of the active site thiol.

The enzyme cathepsin B, whch was the focus of this investigation, will be discussed in greater detail in Chapter 4.

2.5.2 LAMPs

LAMPs are the major protein constituents of late endolysosomal membranes, which is consistent with a putative role in protecting these membranes from hydrolysis and thus preventing leakage of the hydrolases into the cytoplasm. The phagosome, the AVd, the late endosome and the lysosome are all enriched in LAMP proteins. LAMPs are ubiquitously distributed throughout mammalian cells, and make up as much as 50% of the total protein found on 'lysosomal' membranes. LAMP-1 and LAMP-2 are evolutionarily related: both are type I membrane proteins, with a short cytoplasmic tail, a transmembrane region and a large luminal domain. The short (10-11 residue) cytoplasmic tail contains targeting information directing the LAMPs to the late endosome/lysosome (reviewed in Peters and von Figura, 1994). Proteolytic processing of this tail probably ensures that the protein is retained by these organelles (Guarnier *et al.*, 1993). Although LAMPs and MPRs are directed to the same organelles, they use different targeting machinery (Karlsson and Carlsson, 1998).

The large luminal domain of the LAMPs has a high carbohydrate content with 16-20 N-linked-glycosylation sites as well as O-linked-glycosylation sites. The luminal domain of

these proteins also has a Pro-rich hinge region and 4 contiguous disulfide bridges (Peters and von Figura, 1994). The high sialic acid content of their carbohydrate moieties contributes to their low pI, which may be of functional significance. LAMPs may participate in aggregation of the soluble lysosomal contents (Jadot *et al.*, 1997). This could be due to the low pI of the LAMPs, allowing these proteins to behave as cation-exchangers. This putative regulatory mechanism will be discussed in greater detail below. When expressed on the cell surface, LAMPs may also play a role in cell adhesion processes (Silverstein and Febbraio, 1992).

LAMP-1 (Andrejewski *et al.*, 1999) and LAMP-2 (Tanaka *et al.*, 2000) deficient mice have recently been generated. The LAMP-1 deficient mice were viable and fertile, and had lysosomes that had similar properties to control lysosomes. The loss of LAMP-1 appeared to be compensated by increased expression of LAMP-2 (Andrejewski *et al.*, 1999). LAMP-2 mutants, on the otherhand, showed increased mortality when compared to control mice, and showed an extensive accumulation of AV*i*s. This data suggests that LAMP-2 is necessary for maturation of the AV*i* to the AVd (Tanaka *et al.*, 2000). The LAMP-1 and LAMP-2 double mutation is lethal (Andrejewski *et al.*, 1999).

2.5.3 The cysteine transporter

The primary function of endolysosomal proteolysis is to degrade macromolecules for recycling into anabolic reactions. Egress of the products of proteolysis from this sealed environment is largely undertaken by transporters, which have been described for carbohydrate monomers, nucleosides, amino acids and ions (reviewed by Pisoni and Thoene, 1991). Two of the transporters that could be involved in regulating the redox potential of the endolysosomal environment are the cystine and cysteine-specific transporters and the possible role of these in supporting lysosomal proteolysis will be the focus of this discussion.

Disulfide bridges are important in the structural stability of proteins and reduction of these in the endolysosomal environment may increase the rate of proteolysis. Cysteine is believed to be the physiological reducing agent involved (Pisoni and Thoene, 1991). However, reduction of disulfide bridges by cysteine results in the generation of cystine (Lloyd, 1992), which is poorly soluble and crystallises unless removed from the endolysosome. This occurs in the disease, cystinosis, due to a defect in the cystine

transporter (Pisoni and Thoene, 1991). The properties of the cystine transporter have been evaluated *in vitro* using counter-transport and *trans*-stimulation studies, although such studies are complicated by the heterogeneity of 'lysosomal' preparations.

In contrast to other 'lysosomal' transporters, the cysteine-transporter effects a net influx of cysteine *into* 'lysosomes'. When human fibroblasts were incubated with [^{35}S]cystine, at least 50-60% of the total radioactivity taken up by the cells was found associated with 'lysosomes' in the form of cysteine (Pisoni *et al.*, 1990). This uptake occurred rapidly (2-5 min) and the transporter was found to be highly specific. Transport into the 'lysosome' was stimulated when the pH outside was greater than the luminal pH (Pisoni and Velilla, 1995). Cysteine-transport activity has also been described in lysosomal fractions from macrophage and B-cell lymphoma cell lines (Gainey *et al.*, 1996). In the B-cell lymphoma cell line, cysteine transport activity was also detected in antigen processing compartments (Gainey *et al.*, 1996), suggesting that reduction may be a feature of antigen processing.

The 'lysosomal' cysteine transporter, like most endolysosomal transporters, has not been isolated and therefore its intracellular location has not been directly established. However, there is evidence that disulfide reduction occurs in the late endosome compartments rather than in early endosomes (Feener *et al.*, 1990; Collins *et al.*, 1991), suggesting that the cysteine transporter may not be present in early endosomes.

The presence of the cysteine transporter can be interpreted as evidence of reducing conditions within the endolysosomal environment. Indeed, the proteolytic environment may be reducing, but the situation is not as simple as often presented. That reduction does occur is evident from studies on toxins like diphtheria toxin, which requires reducing conditions to be active (Collier and Kandel, 1971; Moskaug *et al.*, 1987). Further, reduction appears to be an essential component of antigen processing (see for example Collins *et al.*, 1991; Merkel *et al.*, 1995), e.g. presentation of the insulin A chain requires that the cysteine residues are in the thiolate form (Hampl *et al.*, 1992). Further, Gainey *et al.* (1996) demonstrated that antigen-presentation of antigens with disulfide bonds, occurs in compartments capable of cysteine-transport. The effect may also be a general one, that reducing conditions enhance substrate proteolysis, which is not limited to antigen processing.

Reducing conditions may affect both proteases and their substrates. It is often assumed that a reducing environment is necessary for the activity of the lysosomal cysteine proteases. However, although isolated cysteine proteases do require reducing agents in order to be active, there is no evidence that these enzymes are oxidised *in vivo* (Wilcox and Mason, 1992; Krepela *et al.*, 1997; 1999). Oxidation may be an isolation artifact. In the reduced, activated, form the isolated enzymes are more stable to neutral pH conditions (Dehrmann *et al.*, 1996). As will be described below, the late endolysosomal environment may not always be acidic and may cycle up to neutral or even alkaline conditions. A reducing environment may serve to stabilise these proteases during the purported pH changes.

Reducing conditions would also affect the substrate proteins. It was found that substrates with disulfide links are cleaved with greater efficiency by lysosomal proteases in the presence of a reducing agent (Kooistra *et al.*, 1982; Mego, 1984). Reducing agents are believed to work synergistically with the proteases by reducing the disulfide links, relaxing the substrate structure and exposing more sites for proteolysis. Thus, a reducing endolysosomal environment does seem to confer several advantages for proteolysis. However, Lloyd (1992) has pointed out that the stoichiometry of the reduction reaction itself does not necessitate the continuous influx of cysteine and has suggested that the cysteine transporter may simply serve an anaplerotic function, replenishing cysteine lost to auto-oxidation. It must also be questioned how endolysosomal proteins containing essential disulfide bridges (e.g. cathepsins and LAMPS) are protected against reduction.

A further question is the effect of (low) pH on the reduction potential within the endolysosomal system. Feener *et al.* (1990) constructed a probe ([^{125}I]tyn-SS-PDL), consisting of [^{125}I]iodotyramine linked to poly-D-lysine by a 3-(propionyldithio)propionic acid spacer, that was acid stable and resistant to proteolysis but sensitive to reduction. These authors found that this probe was only inefficiently reduced within the endolysosomal system and suggested that the Golgi/*trans*-Golgi may be the main site of reduction. In experiments *in vitro*, the probe was inefficiently reduced by cysteine (5 mM) at low pH (Feener *et al.*, 1990). A basis for this result can be found by considering the effect of pH on reduction potential. At fixed cysteine/cystine concentrations, redox potential is inversely proportional to pH so the reduction potential will be more oxidising at

lower pH. Low pH conditions within the endolysosomal system may thus generate a relatively oxidising environment.

How may this evidence for and against a reducing endolysosomal proteolytic environment be reconciled? The extremely rapid sequestration of cysteine by 'lysosomes' (Pisoni *et al.*, 1990), we believe, is inconsistent with a purely anaplerotic function for the cysteine transporter as suggested by Lloyd (1992). Moreover, the experiments describing the synergistic effects of cysteine on proteolysis were all undertaken at acidic pH (Kooistra *et al.*, 1982; Mego, 1984), suggesting that reduction is not precluded by low pH. To keep the reduction potential more or less constant, however, at low pH a higher concentration of cysteine and/or a lower concentration of cystine is required. Perhaps the function of the cysteine and cystine transporters is thus to maintain the redox potential within limits by ensuring an influx of the thiol and efflux of the disulfide.

The cytosolic redox buffer is glutathione and cytosolic GSH/GSSG ratios range from 30:1 to 100:1, which corresponds to a redox potential of *ca.* -221 to -236 mV (Hwang *et al.*, 1992). Protein disulfide bridges are largely formed in the ER secretory pathway by the enzymes Ero1p (in yeast) and enzymes of the protein disulfide isomerase (PDI) family (Frand *et al.*, 2000). The redox buffer in the ER/Golgi secretory pathway is also glutathione but here it is more oxidising than the cytosol, with a GSH/GSSG ratio of 1:1 to 5:1 and a redox potential of *ca.* -160 to -170 mV (Hwang *et al.*, 1992). This is in agreement with the optimum GSH/GSSG ratio for the *in vitro* activity of PDI. However, *in vivo*, Pdi1p (the yeast analogue of PDI) occurs largely in the disulfide form and Frand *et al.* (2000) have argued that this balance point is due to efficient oxidation by Ero1p and relatively inefficient reduction by GSH. In the disulfide form, PDI-type enzymes can act as oxidases, transferring disulfides directly to oxidisable proteins.

An essentially opposite situation may obtain in the endolysosomal system where efficient hydrolysis requires the reduction of protein disulfide bridges. Here the reducing agent is thought to be cysteine but the cysteine/cystine concentration is unknown. Nevertheless, modelling of the Nernst equation (Appendix 1) reveals that to achieve a redox potential of -160 to -170 mV (i.e. the same as in the ER) at pH 5.0 with a cysteine/cystine redox buffer (at 1 mM total half-cystine), a cysteine/cystine ratio of *ca.* 180:1 to 200:1 is required and to achieve -221 to -236 mV (i.e. the same as in the cytosol) requires ratios of 500:1 to 600:1. Thus, the dependence of redox potential on pH, could provide a reason for the existence of

the cysteine transporter. A lysosomal reductase, gamma-interferon-inducible lysosomal thiol reductase (GILT) has been described (Arunachalam *et al.*, 2000), which is optimally active at acidic pH, is activated by cysteine (and not by glutathione) and is capable of reducing protein disulfides. Although details are unknown, it is possible that reduction of protein disulfides may follow a scheme such as shown in Fig. 2.3.

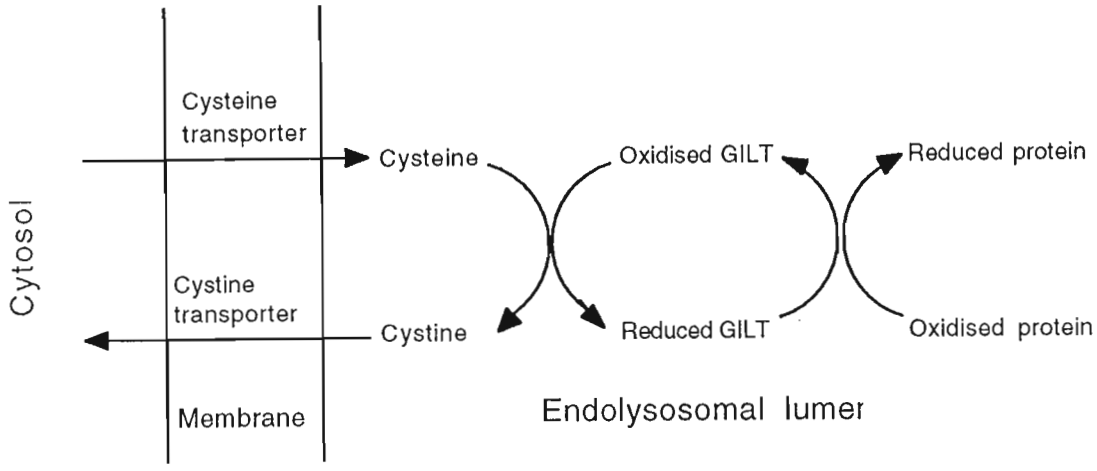

Figure 2.3. A hypothetical scheme for reduction of substrate proteins in the endolysosomal system.

The proposed interposition of GILT between cysteine and oxidised proteins is analogous to the interposition of PDI between oxidants and protein substrates in the ER and could play a similar role in introducing greater efficiency and specificity into the reduction reactions. For example, the fact that the [^{125}I]tyn-SS-PDL probe of Feener *et al.* (1990) was not reduced may be because GILT could not recognise this substrate. Similarly, lysosomal proteases with disulfide links may have structural features that exclude them as substrates for GILT.

Hydrogen peroxide is produced by normal cellular metabolism, and redox recycling drugs (Öllinger and Brunk, 1995). A consequence of having cysteine sequestered in lysosomes is that it may combine, at low pH, with intra-lysosomal iron and hydrogen peroxide, to produce hydroxyl radicals by Fenton reactions (Zdolsek *et al.*, 1993; Brunk *et al.*, 1995; and Garner *et al.*, 1997). Hydroxyl radicals are highly destructive reactive oxygen species and damage lipids, DNA and proteins (Allen, 1991). These reactions could contribute to lysosomal leakage, which could effect processes like apoptosis. The lysosomal membrane is highly enriched with the anti-oxidant α-tocopherol, to a significantly higher degree than even mitochondria (Rupar *et al.*, 1992). This may decrease leakage from the lysosome by inhibiting oxidant damage of the membrane.

2.5.4 Acidification

An acidic luminal environment is important for the following functions:

- Receptor-mediated endocytosis and recycling. Receptors, like the mannose-6-phosphate receptor, require acidic conditions in order to discharge their ligands and recycle.

- Movement/maturation of organelles. ECV formation requires a functional V-ATPase (Clague *et al.*, 1994) suggesting a link between the movement of substrate along the endolysosomal pathway and acidification.

- Activity of membrane transporters. 'Lysosomal' membrane transporters, like the cystine transporter (Gahl and Tietze, 1985), have acidic pH optima that effectively ensures that amino acid transport is from the lysosome to the cytoplasm (Pisoni and Thoene, 1991).

- Activation of lysosomal hydrolases and proteolysis. Many of the lysosomal hydrolases are proteolytically processed to their mature forms under acidic conditions (Hasilik, 1992), some by auto-processing. Further, many of these hydrolases have acidic pH optima and are therefore maximally active under acidic conditions. An acidic environment may also contribute to denaturation of substrate proteins, increasing the rates of proteolysis.

An acidic luminal environment is established by the vacuolar-H^+ATPase (V-ATPase) and a redox chain (Gille and Nohl, 2000), both of which pump protons into the lumen. The V-ATPase is made up of two multi-subunit sectors: V_0, an integral membrane sector and V_1, which is essentially a cytoplasmic sector. The V_0 sector is made up of at least nine subunits (100, 38, 19, 17_6-kDa), and is responsible for proton translocation. The V_1 sector is also made up of at least nine subunits (73_3, 58_3, 40, 34, 33-kDa) and is responsible for the ATPase activity. The ATP binding regions are found on the 73 kDa A-subunits which show a high degree of cooperativity in ATP catalysis (reviewed by Futai *et al.*, 1998). Also found within the A-subunit is a conserved region known as the P-loop. The P-loop has a cysteine residue (Cys 254) which is capable of undergoing thiol/disulfide exchange with Cys 532, allowing for redox regulation of the V-ATPase (Feng and Forgac, 1994). Disulfide

bond formation is believed to induce a conformational change, depressing ATPase activity and inhibiting proton translocation (Feng and Forgac, 1994). This mechanism provides a further link, though of a different type, between redox potential and pH.

Although the V-ATPase is responsible for translocating protons into the endolysosomal lumen environment, regulation of the pH of this environment also depends on other factors. Continuous influx of protons into an organelle would result in the generation of a net positive membrane potential which would inhibit further proton translocation. It is believed that inwardly-directed chloride ion channels, and perhaps cation-channels also, may help regulate the pH of an organelle (reviewed by Futai *et al.*, 1998). The pH of 'lysosomes', two days after isolation, and in the absence of Mg ATP, remains relatively constant. This suggests that 'lysosomal' pH is maintained by a Donnan-type equilibrium (Moriyama *et al.*, 1992). As described above, the early endosome pH is regulated by a Na^+/H^+-exchanger that helps to create an internal positive membrane potential, inhibiting further proton uptake (Fuchs *et al.*, 1989; Cain *et al.*, 1989). The number of V-ATPase molecules found on an organelle may also influence the pH of that organelle.

Given the importance of an acidic environment to the proper functioning of the endolysosomal system, are all lysosomes acidic? Some of the cysteine endoproteases found within the endolysosomal system are capable of working over a broad pH range. In fact, *in vitro*, cathepsin B has a neutral pH optimum against synthetic substrates (Willenbrock and Brocklehurst, 1985a; Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992; Dehrmann *et al.*, 1996). Cathepsin S is also capable of operating at neutral pH (Kirschke *et al.*, 1998). Butor *et al.* (1995) confirmed the late endolysosomal/lysosomal location of two enzymes, sialic acid-specific 9-*O*-acetyl-esterase and glycosyl-*N*-asparaginase, both of which have neutral to alkaline pH optima and further demonstrated that not all lysosomes were acidic. They suggested that the 'lysosomal' pH may cycle between acidic and neutral pHs. At that time, however, it was not known that most proteolysis takes place in late endosomes and not lysosomes (Tjelle *et al.*, 1996; Bright *et al.*, 1997), though it remains to be demonstrated that this is also true for the hydrolysis of the glyco moieties of glycoproteins. The concept of lysosomes as storage organelles was also not fully developed. Nevertheless, it may be assumed that, taken as a whole, the pH of the luminal environment of the endolysosomal pathway may be highly dynamic, allowing for the optimal activity of the different hydrolases found within the system. In the endolysosomal system, a pH cycle

similar to that suggested by Butor *et al.* (1995) may exist as a consequence of a late endosome/lysosome traffic cycle.

2.5.5 Luminal calcium

The role of cytoplasmic calcium in lysosomal homotypic fusion (Bakker *et al.*, 1997), lysosome-plasma membrane fusion (Andrews, 2000), and phagosome-lysosome fusion (Jaconi *et al.*, 1990; Malik *et al.*, 2001) has been well documented. However, the role of *luminal* calcium in regulating proteolysis within these organelles has not been as extensively studied. To the author's knowledge the role of intra-organellar calcium in regulating phagosome function is unknown. Autophagy, however, appears to depend on intracellularly sequestered calcium (Gordon *et al.*, 1993) though the identity of the organelle(s) involved is not known. 'Lysosomes' have been shown to be an intracellular pool for calcium (Haller *et al.*, 1996), and the existence of a lysosomal calcium transporter has been demonstrated (Lemons and Thoene, 1991). As discussed above, hybrid organelle formation is dependent on calcium release from the lumen of late endosomes and lysosomes to facilitate membrane fusion (Pryor *et al.*, 2000). Luminal calcium may also play a role in lysosome reformation from the hybrid organelle (Pryor *et al.*, 2000). The effect that luminal calcium plays on lysosomal proteases has not, to the author's knowledge, been reported.

2.6 Regulation of endolysosomal proteolysis

2.6.1 Are endolysosomal proteases regulated by intra-luminal redox conditions?

A conceivable control mechanism for endolysosomal cysteine proteinases is via regulation of the redox conditions in the intra-luminal environment as discussed above. Both cathepsins B and L are stable in the range pH 4.5 to pH 6.5 and, *in vitro*, require a reducing environment for activity (Kirschke *et al.*, 1998), which is thought to be required to maintain the active site cysteine in a thiol form. However, there is no evidence that these enzymes are oxidised *in vivo*. Labelling of cathepsin B in isolated mouse liver lysosomes with the inhibitor Z-[^{125}I]-Tyr-Ala-CHN$_a$ did not increase with the addition of DTT or cysteine (Wilcox and Mason, 1992). These two reducing agents also did not enhance the activity of cathepsin B in extracts from lung carcinomas and lungs (Krepela *et al.*, 1997; 1999). This suggests that redox regulation of the active site thiol may not occur in the endolysosomal

proteolytic environment. Further, it also suggests that these enzymes may not necessarily be oxidised upon their secretion into the extracellular environment.

Whilst the active site thiols of these enzymes may not be oxidised, it is possible that their ionisation state may be altered. At very low pH, the activity of the cathepsins is depressed. This may be related to a pH-dependent change in the ionisation state from a thiolate (RS^-) to a thiol (RSH) form. However, at low pH cathepsin D would be active (Table 2.2).

A report by Krepela *et al.* (1999) suggested that cathepsin B may be inhibited by low concentrations of the thiol (RSH) form of cysteine, which acts as a competitive inhibitor. The inhibitory effect of the thiol form of cysteine could be reversed by increasing concentrations of the thiolate (RS^-) form, and was less prevalent at higher pH. These authors suggested that cysteine may bind into the S1' subsite of the enzyme, inhibiting its function. This subsite varies between the different cathepsins (Kirschke *et al.*, 1998) and this may not be a general regulatory mechanism for all the enzymes. However, since cathepsin B is the most prevalent endoprotease, regulating its function may significantly affect proteolysis within the endolysosomal system.

A study of the effects of changes in redox potential on the activity of (100% active) cathepsin B, showed that redox potential *per se* had very little effect on the activity (Chapter 5) It is concluded that although the endolysosomal lumen may be reducing, changes in cysteine/cystine-induced redox potentials are probably not involved in regulating endolysosomal proteases. Moreover, an expectation of any regulatory mechanism is that it should include all hydrolases within the system and other endolysosomal hydrolases, e.g. α -glucosidase, have no cysteine residues which could be affected by redox changes.

Unfortunately, the cysteine transporter has not been isolated and its intracellular location, e.g. lysosomes vs late endosomes, and whether it is present in autophagic vacuoles, has not be determined. Thus, at this stage, it is not possible to unequivocally state that reducing conditions are, or are not, a necessary attribute of a proteolytic compartment.

2.6.2 Regulation by pH.

Regulation by pH changes, in contrast to redox changes, could simultaneously influence many enzyme classes. Factors that could regulate the luminal pH include: the redox state of

the cysteine on the P-loop of the V-ATPase, the presence of Cl^- transporters on the organelle, a Donnan-type equilibrium, the number of V-ATPases found on that organelle (reviewed by Futai *et al.*, 1998), and possibly also a membrane redox chain (Gille and Nohl, 2000). What is not clear is how these factors work together to establish the lumen pH. As described above the lumen pH may be dynamic. Within the early endosome, pH appears to be regulated by G-proteins (reviewed by Warnock, 1999), which respond to transporter entry into the early endosome. Also, Na^+/K^+ exchangers help modulate this environment. The (relatively high) pH within this environment allows cathepsin H to function optimally.

Acidification could also contribute to the storage of endolysosomal enzymes by complexation. Kostoulas *et al.* (1997) found that elastase and other enzymes within the azurophil granules of human neutrophils bound to sulfonated glycosaminoglycans by electrostatic interactions at low pH. It was proposed that this may be a storage mechanism for these enzymes. Pryor *et al.* (2000) suggested that lysosome condensation may be similar to secretory granule formation. A feature of secretory granules is that they contain condensed cores of aggregated proteins, a morphology similar to that of lysosomes (Griffiths, 1996). Using bovine pituitary gland cells, it was demonstrated that the granule content proteins and the luminal domains of granule membrane proteins could aggregate at low pH (< pH 5.5). A similar result was obtained for bovine adrenal glands, although aggregation depended on the presence of Ca^{2+}. Proteins destined for constitutive secretion did not aggregate with the granule content and luminal membrane proteins, and this property may serve as a segregation mechanism for those proteins to be stored and those that are constitutively secreted (Colomer *et al.*, 1996).

The aggregation of lysosomal enzymes at pH 4.8 in Chinese hamster ovary (CHO) cells has been described (Buckmaster *et al.*, 1988). This aggregation was disrupted by NaCl, suggesting an electrostatic mechanism. Horseradish peroxidase (HRP) that had been chased into the CHO cells failed to aggregate with the lysosomal enzymes, suggesting that aggregation was specific. Jadot *et al.* (1997) showed aggregation of rat liver lysosomal enzymes at low pH. This aggregation occurred between pH 4.5-5.0, and was mediated by the integral membrane protein LAMP-2. Under these low pH conditions, lysosomal enzymes bind by electrostatic interactions to the LAMP-2 proteins, immobilising them in a matrix. This process was specific for the eleven lysosomal hydrolases assayed during the experiment: a cytosolic extract and bovine serum albumin (BSA) failed to aggregate with

LAMP-2 under the conditions tested. Unlike the aggregation observed in CHO cells (Buckmaster *et al.*, 1988), detergents affected aggregation of the rat liver lysosomal enzymes, suggesting that membrane association with LAMP-2 was vital for aggregation. Lysosomal condensation from the hybrid lysosome/late endosome organelle was shown to be dependent on intra-organellar Ca^{2+} and a functional V-ATPase (Pryor *et al.*, 2000). If this process involved aggregation of the lysosomal hydrolases it would be analogous to the pH and Ca^{2+}-dependent aggregation found in bovine adrenal granules (Colomer *et al.*, 1996). Further, if this process involved LAMP-2 it may specifically aggregate the lysosomal luminal proteins (Jadot *et al.*, 1997), allowing lysosome condensation without contaminating proteins.

A pH-dependent aggregation mechanism could operate to withdraw lysosomal hydrolases out of the late endosome, and/or it could be a feature of the lysosome itself. An advantage of this complexation mechanism, as opposed to a purported redox-dependent storage mechanism, is that it could encompass several different types of lysosomal hydrolases (Jadot *et al.*, 1997). Further, as described by Griffiths (1996), under the electron microscope lysosomes appear to be small, highly dense organelles, a morphology that could be explained by aggregation of their luminal hydrolases.

The concept of lysosomal enzymes aggregating by electrostatic interactions is a fairly old one (see for example Henning *et al.*, 1973). However, the acceptance of this as a mechanism for regulating and storing lysosomal hydrolases has been limited. This is partly because erroneously low pH optima were assigned to some hydrolases (see Dehrmann *et al.* 1995), which by chance coincided with the low pH of lysosomes where the bulk of substrate hydrolysis was thought to occur. Subsequently, it has been found that most proteolysis occurs in late endosomes which have a pH closer to the revised pH optima of lysosomal proteases. The hypothesis that lysosomal hydrolases may be stored by low pH-induced aggregation does not exclude the possibility that late endosomes may fluctuate between alkaline and acidic pH values, allowing for the activation of different hydrolases with individual pH optima (Butor *et al.*, 1995). The lysosome with its low pH may complex the lysosomal hydrolases, effectively storing them in a precipitated matrix.

2.7 Conclusions

Complicating the whole field of endolysosomal proteolysis is the lack of a consistent nomenclature, especially concerning the definition of the 'lysosome'. To many authors a 'lysosome' is any organelle containing 'lysosomal' hydrolases. However, this simple definition may have outlived its usefulness and it may be more usefully replaced by a functional definition, e.g. that lysosomes are organelles mainly for the storage of 'lysosomal' hydrolases in a deactivated state. To become functional, 'lysosomal' hydrolases must be injected into a late endosome, a phagosome or an autophagosome, which are the organelles within which hydrolysis largely occurs. This concept of a lysosome is not too different from the original concept of a "primary lysosome" (De Duve 1983). In fact, the only substantial difference is that it was perhaps not originally known that "primary lysosomes" themselves are not hydrolytically active but that activity is largely manifest in "secondary lysosomes", i.e. late endosomes, phagosomes and autophagosomes.

Another modern, functional, definition of a lysosome is that it is, "the terminal organelle in the endolysosomal pathway and is devoid of recycling receptors" (Griffiths, 1996). In the early definition of a "primary lysosome" this was envisioned as being a vesicle, containing newly synthesised enzymes, which budded off the Golgi apparatus (De Duve 1983). Griffiths (1996) has noted that lysosomes are indeed vesicle-sized, so in microscopy studies they may easily be confused with 'lysosomal' enzyme carrier vesicles, which have a similar cargo. However, the latter are devoid of MPRs.

Many hydrolytic organelles (secondary lysosomes) may be assembled *de novo* when required and this gives an opportunity to determine a minimal requirement for hydrolysis. A common theme applicable to the endolysosomal system, phagosomes, and autophagosomes is that enzymes are imported by fusion with lysosomes (where they are stored in a largely inactive form), acidification is effected by importation of a V-ATPase, and egress of products is effected by importation of membrane transporters. Some pathogens exploit the endolysosomal system and escape hydrolysis by blocking the assembly of a complete functional hydrolytic compartment.

Reactions between enzymes and their substrates, in solution, are described by the reaction ,

$$E + S \rightleftarrows ES \rightarrow E + P \qquad (1)$$

and can be analysed by conventional kinetic methods. Such enzymes are often regulated by soluble effector molecules, typically inhibitors in the case of non-allosteric enzymes. Enzyme reactions in the endolysosomal system, however, can be described by the model, given in Fig. 2.1. There are five possible points of regulation within the endolysosomal system: i) at the point of acquisition of substrate by the endolysosomal system, ii) at the meeting of E and S by fusion of their respective compartments, iii) at the establishment of conditions supporting proteolysis (or not) within the (fused) organelle, (iv) at the point of egress of product from the system and (v) the possibility of an enzyme being retained in a lysosome for a greater or lesser period (perhaps in an inactive form, but in any event separate from substrate).

The model described by Fig. 2.1 can explain why there are a number of endolysosomal compartments (to optimise luminal conditions to favour certain reactions). Steps (i) and (ii) are regulated by signalling networks which may control the rate-limiting steps in endolysosomal hydrolysis. They may exert their influence through molecules that interface between the signalling and endocytic pathways. Future research efforts could be directed at attempting to unite the signalling networks, the regulation of endolysosomal fusion reactions, and the components that affect the luminal endolysosomal proteolytic environments [steps (iii) and (v)] into an integrated model for proteolysis.

CHAPTER 3

MATERIALS AND METHODS

This chapter will describe fundamental methods that were routinely used during the course of this research project. In addition, specialised or novel procedures that would hinder the structure of subsequent chapters are described here.

3.1 Materials

Most of the common chemicals used during this study were from BDH, Merck or Boehringer Mannheim and were of the highest purity available. Coomassie brilliant blue G-250 was from Merck. Acrylamide, N,N'-methylbisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), dioxane and dimethylformamide were from BDH. Dialysis tubing, 7-amino-4-methyl coumarin, dithiothreitol (DTT), L-trans-epoxysuccinyl-leucylamido(4-guanidino) butane (E-64), activated CH-Sepharose, 2,2′ dipyridyl disulfide and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), pepstatin A, aminohexylagarose, N-hydroxysuccinimide and dicyclocarbodiimide, as well as the protease substrates Cbz-R-R-AMC and Cbz-F-R-AMC, haemoglobin, bovine serum albumin (BSA), lysozyme and azocasein, were from Sigma (St. Louis, MO). The cation exchange chromatography gel, S-Sepharose, was from Pharmacia Biotech, Uppsala, Sweden. The Gly-Phe-GlySc ligand was from Bachem (Switzerland). Cysteine (free base, >99% pure) was from Fluka Biochemika (Switzerland). UltraFuge ultrafiltration centrifuge filters were from Micron Separations Inc. (Westbro, MA). Distilled H_2O (dist.H_2O) was produced by a Milli-RO® 15 Water Purification System (Millipore, Marlboro, USA). Distilled, deionised water (ddH_2O) was produced by a Milli-Q® Plus Ultra-Pure water system (Millipore, Marlboro, USA). The minimum resistivity of the ddH_2O was 18 MΩ

3.2 Protein assays

The Bradford dye-binding assay (Bradford, 1976), as modified by Read and Northcote (1981), was used for protein quantification.

3.2.1 Bradford dye-binding assay

The Bradford dye-binding assay is based on the binding of the dye, Coomassie brilliant blue G-250, to protein. The assay allows for rapid, accurate and reproducible protein quantification. In addition, the assay requires small amounts of sample, and is resistant to interference by chemicals commonly used during protein purification such as Tris, ethylene diamine tetra-acetic acid (EDTA), Triton X-100 and sodium dodecyl sulfate (SDS).

The Coomassie brilliant blue G-250 dye exists in three forms, an anionic blue form, a neutral green form and a cationic red form. The cationic form has an absorption maximum at 470 nm. Upon binding to protein the cationic form is converted to the blue species which has an absorption maximum at 595 nm. This shift in absorbance maximum allows for spectrophotometric quantification of protein. The extinction coefficient at 595 nm of the dye-protein complex is much greater than that of the unbound dye and is responsible for the high sensitivity of the assay.

A large proportion of the dye binding energy is due to non-ionic interactions. This results in variation in the binding properties of the dye with different proteins. In order to minimise this variation, Read and Northcote (1981) modified the acid/alcohol ratios and increased the dye concentration used in the dye reagent. The micro-assay of Read and Northcote (1981) was used in this study for the determination of 1-5 µg of protein.

3.2.1.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). This solution was made up to 500 ml with dist.H_2O and stirred for 30 min. The resulting solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle. Visual checks for precipitation were made prior to use and, if precipitation was observed, a new batch of reagent was made up.

Standard protein solution. A 0.1 mg/ml ovalbumin solution was made up in dist.H_2O.

3.2.1.2 Procedure

Samples were diluted to 50 µl with dist.H_2O, dye reagent (950 µl) was added, and the solution was mixed and allowed to stand for 2 min for colour development. The absorbance

was read at 595 nm against appropriate blanks. Plastic microcuvettes (1 ml) were used, as the dye reagent binds to glass (Bradford, 1976). The blue stained plastic microcuvettes could easily be cleaned with diluted sodium hypochlorite. A standard curve, relating absorbance at 595 nm to 0-50 µl (0-5 µg) of the standard protein was constructed by linear regression analysis of the standard curve data.

3.3 Concentration of enzyme samples

Many of the enzyme isolation procedures used during this study required prior concentration of the dilute protein samples. An inexpensive and simple method of concentrating samples was by dialysis against sucrose or polyethylene glycol (PEG). Samples were placed in dialysis tubing with a cut-off of 12 kDa and dialysed against either sucrose or PEG. Osmotic pressure results in the continuous efflux of water and buffer ions from the dialysis bag, concentrating the sample. PEG (20 kDa) tends to interfere with protein quantification and enzyme activity assays but, unlike sucrose, does not enter the dialysis bag. When necessary, these two concentration methods were supplemented using concentration with UltraFuge ultrafiltration centrifuge filters. The UltraFuge filters are divided into two chambers separated by a filter with a M_r cut-off of 10 000. Dilute samples were loaded into the upper chamber and the UltraFuge filters were centrifuged (2000 x g, 30 min, 4°C). Under the centrifugal forces, water and buffer ions pass through the membrane, while the target proteins, which were usually between 30-50 kDa, are retained. This method of concentration has none of the disadvantages associated with sucrose or PEG. These filters did prove to be problematic, however, often rupturing at very low centrifugal forces.

3.4 Electrophoretic techniques

3.4.1 Tris-tricine SDS-PAGE

The Tris-tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system was designed to resolve smaller proteins (< 100 kDa) which are sometimes poorly resolved by conventional Laemmli SDS-PAGE. In this system, tricine rather than glycine is used as the trailing ion in the stacking phase. Tricine, and the higher pH of the stacking phase, causes an increase in the mobility of the protein relative to the trailing ion, allowing

for the separation of low molecular weight protein-SDS complexes (Schägger and von Jagow, 1987).

3.4.2 Reagents

Monomer solution [49.5% (m/v) acrylamide (T), 3% (m/v) N,N'-methylene-bisacrylamide (C)]. Acrylamide (49.5 g) and N,N'-methylene-bisacrylamide (3.0 g) were dissolved in dist.H_2O and made up to 100 ml. This solution was filtered through Whatman No. 5 paper and stored at 4°C in the dark.

Gel buffer [3 M Tris-HCl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) was dissolved in 200 ml dist.H_2O, and adjusted to pH 8.45 with HCl. 10% (m/v) SDS (6 ml) was added and the solution made up to 250 ml.

10 % (m/v) SDS. SDS (10 g) was dissolved in dist.H_2O and made up to 100 ml.

Anode buffer [0.2 M Tris-HCl, pH 8.9]. Tris (24.22 g) was dissolved in 950 ml of dist.H_2O, adjusted to pH 8.9 with HCl and made up to 1 litre.

Cathode Buffer [0.1 M Tris-HCl, 0.1 M Tricine, 0.1% (m/v) SDS, pH 8.25]. Tris (12.1 g), Tricine (17.9 g) and 10% (m/v) SDS (10 ml) were made up to 800 ml with dist.H_2O and the pH checked and adjusted if necessary. The buffer was made to a final volume of 1L.

Treatment buffer [125 mM Tris-HCl, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.01% (m/v) Serva blue G, pH 6.8]. Stacking gel buffer (2.5 ml), glycerol (2 ml), 10% (m/v) SDS (4 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dist.H_2O. Serva blue G tracking dye [0.01% (m/v)] was added. For non-reducing SDS-PAGE, 2-mercaptoethanol was omitted. Samples were boiled for 2 min in treatment buffer before electrophoresis.

3.4.3 Procedure

The compositions of the resolving and stacking gels used to construct the Tris-tricine system are described in Table 3.1. The gels were run at 80 V with unlimited mA, and stopped when the Serva blue dye reached the bottom of the gel. The gels were stained as described in Section 3.5.

Table 3.1 Preparation of resolving and stacking gels for Tris-tricine SDS-PAGE.

Reagents	Resolving gel 10% T, 3% C	Stacking gel 4% T, 3% C
Monomer (49.5% T, 3% C)	3.6 ml	0.5 ml
Gel Buffer	6 ml	1.5 ml
dist.H_2O	8.4 ml	4 ml
Ammonium persulfate	60 µl	30 µl
TEMED	6 µl	12 µl

3.5 Silver staining of electrophoretic gels

Protein bands were visualised using silver staining, which is at least 50-100 times more sensitive than conventional Coomassie staining and can detect protein in the nanogram range. The basis of silver staining is the complexation of silver ions to the ionic side-chains of amino acids. This binding is responsible for the sensitivity of the technique. The silver-amino acid complex is visualised by reduction with formaldehyde. Reducing reactions are strongly favoured under alkaline conditions and these conditions were generated using Na_2CO_3. The colour produced upon reduction of the silver-amino acid complex depends on the moiety that the silver ion binds to and the length of the bond and its configuration (Nielson and Brown, 1984).

Sodium thiosulfate complexes and removes uncomplexed silver ions present in the gel that may contribute to background staining. Treatment of gels with sodium thiosulfate decreases the levels of background staining without impairing sensitivity (Blum *et al.*, 1987).

3.5.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.2% (v/v) formaldehyde]. Methanol (100 ml), glacial acetic acid (24 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O.

Wash solution [50% (v/v) ethanol]. Absolute ethanol (100 ml) was made up to 200 ml with dist.H_2O.

Pre-treatment solution [0.02% (m/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.1 g) was made up to 500 ml with dist.H_2O.

Impregnation solution [0.08 % (m/v) $AgNO_3$, 0.03 % (m/v) formaldehyde]. $AgNO_3$ (0.4 g) and 37% formaldehyde (0.15 ml) were made up to 500 ml with dist.H_2O.

Development solution [6% (m/v) Na_2CO_3, 0.0004% (m/v) $Na_2S_2O_3.5H_2O$, 0.02% (v/v) formaldehyde]. Na_2CO_3, (12 g), pre-treatment solution (4 ml) and 37% formaldehyde (0.1 ml) were made up to 200 ml with dist.H_2O.

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

3.5.2 Procedure

All steps were carried out at room temperature in clean glass containers. All glass containers, including volumetric flasks, were washed with dist.H_2O and ddH_2O. After electrophoresis, gels were soaked in fixing solution for either 1 h or overnight. Overnight fixing significantly reduced background staining. Following fixation, the gels were treated with wash solution (3 x 20 min). These washing steps allowed for the treatment of gels with acid labile $Na_2S_2O_3.5H_2O$, and could be increased to 30 min washes to reduce background staining. The gels were treated with pre-treatment solution (1 min), rinsed with ddH_2O (3 x 20 s) and soaked in impregnation solution (20 min). After rinsing with ddH_2O (3 x 20 s), the gel was immersed in development solution until bands became visible. The gel was rinsed with ddH_2O, treated with stop solution, and stored in a sealed plastic bag at 4°C.

3.6 Protein fractionation using three phase partitioning (TPP)

TPP is an upstream purification method used to selectively concentrate proteins. Tertiary butanol is completely miscible with water, but upon the addition of sufficient ammonium sulfate the solution partitions into two phases; an upper t-butanol phase and a lower aqueous phase. If proteins are present within the solution, they may, depending on the

ammonium sulfate concentration, precipitate into a third phase between the upper t-butanol and lower aqueous phases (Pike and Dennison, 1989a).

An advantage of TPP compared to most conventional ammonium sulfate precipitation methods is that the precipitate is essentially free of contaminating salt, which remains in the aqueous phase. The re-dissolved precipitate can therefore be directly applied to an ion-exchange column without the need for desalting steps. A further advantage of TPP is that lipids and other hydrophobic compounds are extracted into the *t*-butanol phase. TPP does tend to denature some oligomeric proteins. This property was exploited in the purification of cathepsin D from bovine spleen where haemoglobin is a major contaminant (Jacobs *et al.*, 1989).

The mechanism underlying TPP has been described in detail by Dennison and Lovrien (1997). TPP precipitation of proteins is a result of the co-operative effects of ammonium sulfate and t-butanol. The sulfate ion effects protein precipitation by six interdependent mechanisms: ionic strength effects, kosmotrophy, cavity surface tension enhancement, dehydration, exclusion-crowding, and ionic interactions with cationic groups on the protein. The latter effect is prevalent at low ammonium sulfate concentrations, when the concentration of ammonium sulfate is in the range 0.1 to 0.2 M. These ionic interactions could explain the strong pH dependency of the method. At higher concentrations, 0.4 to 4 M sulfate, ionic strength effects, kosmotrophy, cavity tension enhancement, dehydration and exclusion-crowding come into operation. The net effect of these factors is the conformational shrinkage and contraction of a protein, promoting protein co-crystallisation. Further, this tightening of protein conformation stabilises protein structure, preventing denaturation.

The remarkable protective properties of ammonium sulfate are reinforced by t-butanol. *t*-Butanol behaves as a kosmotrope and a crowding agent, promoting protein structure and enhancing the salting out effect of ammonium sulfate. *t*-Butanol is able to fulfil these co-solvent functions at room temperatures, whereas C_1 and C_2 co-solvents (like ethanol) require low temperatures. The flotation of a protein into the third phase (between the *t*-butanol and aqueous phases) is due to an increase in the relative butanolation and concomitant dehydration of the protein. These effects, mediated by ammonium sulfate, increase the buoyancy of the protein, causing it to float above the salt-containing aqueous

layer. Butanolation can however, cause denaturation of oligomeric proteins by promoting non-native alpha helical conformations in protein structure (Pike and Dennison, 1989a).

TPP has been used to purify the lysosomal proteinases cathepsins L (Pike and Dennison, 1989b), D (Jacobs *et al.*, 1989), S and B (Meinesz, 1996). In this study TPP was used to purify bovine liver cathepsins B and D. A modified homogenisation step was also used (described in greater detail in Section 4.1) in this study. In this procedure, *t*-butanol was added to a final volume of 30% to the homogenisation buffer. Thus, within the initial homogenate, protease activity was suspended (Dennison *et al.* 2000). TPP was effected at a later stage during the purification, and the enzymes returned to full activity.

3.6.1 Procedure

t-Butanol was mixed into the homogenisation buffer to a final volume of 30% (v/v). The volume of t-butanol required was calculated from the equation:

$$y = (0.3/0.7)\,x$$

where y = volume of *t*-butanol

x = volume of buffer

t-Butanol crystallises below 25°C and was therefore warmed to 30°C prior to use. To fractionate the *t* -butanol containing mixture, solid $(NH_4)_2SO_4$ [% (m/v) based on the total volume of the mixture including *t*-butanol] was added to the mixture and dissolved by stirring. This mixture was centrifuged (8000 x g, 10 min, 4°C) in a swing-out rotor, leaving a firm button of precipitate between the t-butanol and aqueous phases. If necessary, after removal of the precipitate, further $(NH_4)_2SO_4$ was added and the solution centrifuged as before. This allowed a cut of the protein of interest to be made from the total protein content of the solution. The precipitate was dissolved in an appropriate buffer, centrifuged (15 000 x g, 10 min, 4°C), and filtered through Whatman No. 4 filter paper to remove undissolved protein. In most cases (in this study), the precipitates were infused with *t*-butanol, which had to be dialysed out.

In conventional TPP, the procedure is very similar to the modified method described above, except that *t*-butanol is usually added after homogenisation.

3.7 Sepharose-Ahx-Gly-Phe-GlySc matrix

Several affinity columns have been described for isolating cysteine proteases, including aminophenylmercuric and Gly-Gly-(OBzl)Tyr-Arg ligand-based columns. These affinity resins either have problems of specificity, and/or the eluted enzymes are active, and thus unstable due to the possibility of autodigestion. An affinity column for cathepsin B purification, Sepharose-Ahx-Gly-Phe-GlySc, was described by Rich *et al.* (1986). Cathepsin B is inhibited by the Gly-Phe-GlySc peptide with a K_i of 63 μM and thus binds strongly to the affinity matrix. However, cathepsin H does not bind to the column, and the binding of cathepsin L to the column is essentially irreversible. Cathepsin B is eluted from the column with 2,2′ dipyridyl disulfide, which binds to the active site thiol, trapping the enzyme in an inactive state (Rich *et al.*, 1986). The eluted enzyme can be rapidly activated with reducing agent when needed. Thus, with this column, cathepsin B can be isolated with great specificity and stability.

3.7.1 Reagents

1 mM Hydrochloric acid. 57 μl of HCl was made up to 500 ml with ddH_2O.

0.1 M Sodium carbonate buffer, pH 8.2. NaH_2CO_3 (4.2 g) was dissolved in 400 ml of ddH_20, adjusted to pH 8.02, and made up 500 ml.

50% methanol. 50 ml of methanol was dissolved in, and made up to 50 ml with, ddH_2O.

6% ethanolamine. 0.6 ml ethanolamine made up to 10 ml with ddH_2O.

0.1% sodium azide. NaN_3 (0.1g) was dissolved in ddH_2O (100 ml).

3.7.2 Procedure

Activated CH-Sepharose was swollen overnight at 4°C in 1 mM HCl. The gel was washed three times with 0.1 M sodium carbonate (pH 8.02). The Gly-Phe-GlySc peptide (25 mg) was mixed with 50% methanol (5 ml) and sodium carbonate buffer (3 ml). This mixture was added to the activated CH-Sepharose 4B gel and agitated overnight at 20°C. The gel was collected on a glass funnel and washed with 50% methanol, followed by ddH_2O. The gel was collected and agitated for four hours at 20°C, washed with several changes of ddH_2O and stored in 0.1% NaN_3.

3.8 Pepstatin aminohexyl affinity resin

The fungally derived aspartic proteinase inhibitor pepstatin A (isovaleryl-Val-Val-Sta-Ala-Sta), binds to cathepsin D with a strong, stable and reversible interaction. This interaction was exploited to purify cathepsin D by affinity chromatography. The pepstatin aminohexyl affinity resin used in this study was prepared according to the method of Murakami and Inagami (1975).

3.8.1 Procedure

Pepstatin A (21.4 mg) was esterified with N-hydroxsuccinimide (3.6 mg) in the presence of dicyclohexylcarbodiimide (6.45 mg). The entire activation reaction was effected in dimethylformamide (1.25 ml) for 22 h (4°C). Aminohexyl agarose (2 g suction dry weight) was suspended in dioxane (3.5 ml) and added to the activated pepstatin solution. The coupling reaction was allowed to occur in a desiccator with gentle stirring (24 h, room temperature). The gel was filtered and washed with a dimethylformamide/dioxane mixture (1:2 v/v, 100 ml), followed by a wash with 1 M NaCl. Washed gel was poured into a glass column (i.d. = 1.5 cm) and stored at 4°C in 1 M NaCl with sodium azide (0.01% w/v) preservative.

3.9 Spectroscopic assay of cysteine

Gaitonde (1967) found that ninhydrin reacted specifically with cysteine at low pH. Other biothiols, like GSH, and other amino acids did not react with the reagent. This reaction formed the basis for a rapid assay of cysteine. Empircally it was found that the assay was not accurate for thiol masses > 0.5 µmoles.

3.9.1 Reagents

Acid ninhydrin reagent [25 mg/ml ninhydrin, 60% acetic acid, 40% HCl]. Ninhydrin (0.25 g) was dissolved in acetic acid (6 ml) and HCl (4 ml) and was stirred at room temperature for 20-30 min. The reagent was always made up just before use.

Thiol diluent solution [5% perchloric acid]. Perchloric acid (5 ml) was dissolved in ddH_2O, and made up to a final volume of 100 ml.

95% ethanol. Absolute ethanol (95 ml) was made up to 100 ml with ddH_2O.

3.9.2 Procedure

Cysteine solutions containing a range of cysteine concentrations (0.1-0.5 µmoles in perchloric acid to prevent oxidation; 0.5 ml) were mixed with acetic acid (0.5 ml) and acid ninhydrin reagent (0.5 ml). The mixtures were heated (95°C, 10 min) resulting in the formation of an intense pink color. The mixes were cooled and diluted to 10 ml with 95% ethanol. Absorbances of the solutions were read at 560 nm and a standard curve (r^2 = 0.996) was constructed using triplicate determinations for each cysteine concentration .

3.10 Quantification of thiol groups on proteins

Ellman's reagent or 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB) provides a highly sensitive method of quantifying thiols in solution. However, protein thiol groups often manifest variable reactivity toward the reagent, presumably due to steric factors. Habeeb (1975) described a method whereby the thiol groups on proteins could be quantified, by first denaturing the protein to expose these groups.

3.10.1 Reagents

Denaturation solution [2% SDS, 0.5 mg/ml EDTA in 0.08 M Na-phosphate buffer, pH 8.0]. $NaH_2PO_4.H_2O$ (12 g), EDTA (0.5 g) and SDS (20 g) were added to 800 ml of ddH_2O, adjusted to pH 8.0 with NaOH, and made up to 1 litre.

0.1 M sodium phosphate buffer pH 8. $NaH_2PO_4.H_2O$ (7.94 g) was dissolved in 450 ml of ddH_2O, adjusted to pH 8.0 with NaOH, and made up to 500 ml.

DTNB reagent [10 mM DTNB in 0.1 M sodium phosphate, pH 8.0]. DTNB (0.04 g) was dissolved in 0.1 M sodium phosphate buffer (10 ml) and stored in an amber bottle in the dark.

3.10.2 Procedure

It was essential that all glassware be free of thiol contamination, and glassware was therefore washed with chromic acid and several rinses of ddH_2O. The test protein solution

(0.01-0.04 μmoles) was diluted with denaturation solution to a final volume of 6 ml. To 3 ml of this solution DTNB (0.1 ml) was added and mixed in. The reaction was developed in the dark for 10 min. To the remaining 3 ml, sodium phosphate buffer (0.1 ml) was added to create a "protein-blank". A "reagent blank" was created by mixing denaturation solution (3 ml) and DTNB (0.1 ml) and incubating the solution in the dark for 10 min. The absorbance of the protein-DTNB mix was determined at 410 nm against the "protein-blank" to give an apparent absorbance. The "reagent-blank" was subtracted from this value to give a net absorbance with an $\varepsilon = 13\ 600\ M^{-1}cm^{-1}$ (Habeeb, 1975).

3.11 Fluorometric assay for cathepsin B activity

The fluorometric substrate Cbz-R-R-AMC has proved to be ideal for cathepsin B. The AMC leaving group is non-toxic, and is sensitive enough for the detection of picomolar amounts of enzyme. Cathepsin B favours an arginine residue at the P1 position, where it can be electrostatically anchored to a glutamic acid residue in the S1 position (Musil *et al.*, 1991). While cathepsin B can cleave the substrates Cbz-R-AMC and Cbz-F-R-AMC, these also can be cleaved by cathepsins H and L, respectively. Both cathepsin H and cathepsin L, and in fact most of the other mammalian cysteine proteases, cannot hydrolyse Cbz-R-R-AMC, making it a relatively specific substrate for cathepsin B (Barrett and Kirschke, 1981). Two assays were used in this study, a microassay for monitoring the enzyme purification, and a macroassay for more sensitive readings.

3.11.1 Reagents

Cathepsin B assay buffer/activator [4 mM Na_2EDTA, 0.02% NaN_3, 5 mM dithiothreitol in 0.1 M Na-phosphate buffer, pH 6.0]. NaH_2PO_4 (6.90 g), Na_2EDTA (0.93 g) and NaN_3 (0.1 g) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml. Immediately before use, dithiothreitol was added to 5 mM (i.e. 0.51 g/500 ml).

1 mM Cbz-R-R-AMC substrate stock solution. Cbz-R-R-AMC (3.1 mg) was dissolved in dimethyl sulfoxide (DMSO) (5 ml), divided into 100 μl aliquots and stored at -20°C. When required, this stock was diluted in ddH_2O to a working concentration of 40 μM (i.e. 40 μl of the stock solution was made up to 1 ml with ddH_2O).

Assay diluent [0.1% Brij]. Brij (0.1 g) was dissolved in ddH_2O and made up to 100 ml.

1 mM 7-amino-4-methylcoumarin standard solution. 7-amino-4-methylcoumarin (1.8 mg) was dissolved in DMSO (10 ml) and stored in a light-proof bottle at -20°C. Appropriate dilutions of the stock solution were made in dist.H_2O.

3.11.2 Procedure

Microassay. Buffer/activator (75 µl) was added to the enzyme sample (10 µl) in a white Fluronunc maxisorp microtitre plate. This mixture was incubated at 37°C for 2 min and substrate solution (25 µl) was added in. After 10 min at 37°C, the fluorescence of the liberated 7-amino-4-methylcoumarin was measured in a fluorescence microplate reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm. Enzyme activity was described as arbitrary fluorescence units.

Macroassay. The enzyme sample was diluted to 125 µl and buffer/activator (750 µl) added in. The mixture was incubated for 2 min at 37°C to activate the enzyme and substrate solution (125 µl) was added in. The fluorescence of liberated 7-amino-4-methylcoumarin was measured continuously for 10 min in a Hitachi F-2000 spectrofluorometer with excitation at 370 nm and emission at 460 nm. Enzyme activity was described by the SI unit for enzyme activity, the katal.

Quantification of liberated 7-amino-4-methylcoumarin. Standard curves relating fluorescence emitted at 460 nm to the concentration of liberated 7-amino-4-methylcoumarin were constructed on the basis of the micro and macro assays. In each case dist.H_2O was used in place of the enzyme sample. The buffer/activator/dist.H_2O mixture was pre-incubated at 37°C for 2 min. An appropriate dilution of the standard 7-amino-4-methylcoumarin solution was added, in place of the substrate solution, and the fluorescence of the solution was determined after 10 min at 37°C. In each case, linear regression analysis of the curves showed a strong correlation with r^2 of 0.991 and 0.996 for the micro and macroassay respectively. The following standard curves were determined using the results obtained from the assays:

$$x = (y - 48.614)/0.181 \text{ for the microassay}$$

$$x = (y - 8.609)/0.536 \text{ for the macroassay}$$

where x = concentration (n moles) of 7-amino-4-methylcoumarin, and y = fluorescence of the solution. For routine enzyme assays, the enzyme activity was described as arbitrary fluorescence units.

3.12 Assay for cathepsin D

The haemoglobin assay developed by Anson (1939) was used in this study to measure the activity of cathepsin D. Cathepsin D hydrolysis of haemoglobin generates TCA-soluble peptides which can be determined spectrophotometrically. Synthetic substrates have been developed for cathepsin D. These substrates have been found to be relatively insensitive (Pohl *et al.*, 1983) or, in the case of the substrate F-A-A-F(NO_2)-F-V-L-OM4P (Agarwal and Rich, 1983), too expensive. During this study an attempt was made to use fluorescein-haemoglobin as a substrate (De Lumen and Tappel, 1970). This was expected to be a trade-off between the synthetic and the native haemoglobin assays. However, the rapid quenching of the fluorescein fluorophore resulted in unacceptably high errors, and the assay was not adopted.

3.12.1 Reagents

5% (m/v) Haemoglobin substrate. Bovine haemoglobin powder (0.5 g) was dissolved in dist.H_2O (10 ml) with magnetic stirring.

Assay buffer [250 mM sodium citrate, 0.02% NaN_3, pH 3.2]. Citric acid (26.3 g) and NaN_3 (0.1 g) were dissolved in about 450 ml of dist.H_2O, adjusted to pH 3.2 with NaOH and made up to 500 ml.

5% (m/v) Trichloroacetic acid. Trichloroacetic acid (5 g) was dissolved in dist.H_2O and made up to 100 ml.

3.12.2 Procedure

Enzyme sample (26 µl), assay buffer (506 µl) and substrate (133 µl) were mixed together in a polyethylene microfuge tube and incubated at 37°C for 30 min. After 30 min, a sample (300 µl) was removed and added to a polyethylene microfuge tube containing TCA (240 µl). A reaction blank was created by replacing the enzyme sample with dist.H_2O (26 µl). Insoluble protein was precipitated by centrifugation on a bench-top microfuge (48 x g,

5 min, RT). The absorbance of the supernatant was read against the blank supernatant in a 1 ml quartz cuvette at 280 nm. One unit of enzyme activity was defined as the quantity of enzyme producing an increase in absorbance of 1.0 in excess of the control in 60 min.

3.13 Active site titration of cathepsin B

Active site titration of cathepsin B was undertaken to determine the percentage of active enzyme present in an isolated sample. Trans-epoxysuccinyl-leucylamido(4-guanidino) butane, or E-64, is a powerful irreversible inhibitor of cysteine proteinases, originally isolated from *Aspergillus japonicus* (Hanada *et al.*, 1978). E-64 binds to cathepsin B in a 1:1 molar ratio. Thus the concentration of active cathepsin B can be determined from the minimum concentration of E-64 required to completely extinguish enzyme activity (Barrett and Kirschke, 1981).

E-64 binds to the S subsites of the enzyme and nucleophilic attack by the active site thiol on the oxirane ring at the C2 position of the inhibitor results in the formation of an irreversible thioether bond. The binding of E-64 and E-64 analogues to the active site of cathepsin B shows a marked pH and ionic strength dependence. The binding of the inhibitor is more effective at lower pH, possibly due to the interaction of the carboxylate group of the inhibitor with the two protonated histidine residues (110 and 111) of the occluding loop. High ionic strength conditions, in the region of 50-150 mM NaCl, disrupt the electrostatic interactions of the inhibitor with the S subsites of the enzyme. The sensitivity of the enzyme to these conditions could account for the differences in kinetic constants obtained in different laboratories (Schaschke *et al.*, 1997). The pH and ionic conditions used for active site titration were as described by Barrett and Kirschke (1981). Active site titration was routinely used to determine the absolute concentration of cathepsin B used in the assays described in Chapter 5, as well as to monitor changes in enzyme activity during enzyme storage.

3.13.1 Reagents

10 mM E-64 stock solution. E-64 (1.8 mg) was dissolved in DMSO (500 µl). Suitable dilutions of the inhibitor were made in Ultrapure Milli-Q water. The dilutions used were based on the concentration of the isolated enzyme (a M_r of 28 000 was assumed for cathepsin B).

3.13.2 Procedure

The active site titration of cathepsin B was adapted from Barrett and Kirschke (1981). Enzyme sample (25 µl), buffer/activator (50 µl) (Section 3.11.1) and an appropriate dilution of E-64 were incubated at 37°C for 30 min to allow the binding reaction to go to completion. All reactions were carried out in triplicate. After 30 min, aliquots from each tube was assayed (Section 3.11) and a graph of activity versus E-64 concentration constructed. The absolute concentration of active enzyme (E_o) was determined by extrapolating the linear portion of the graph to the E-64 concentration axis.

CHAPTER 4

ISOLATION OF CATHEPSIN B AND CATHEPSIN D

4.1 Cathepsin B

The endolysosomal cysteine proteinase, cathepsin B [EC 3.4.22.1], is ubiquitously distributed throughout mammalian cells and its main role appears to be in protein turnover. Cathepsin B also plays important roles in bone resorption and antigen and hormone processing. It has been implicated in pathological conditions like rheumatoid arthritis, tumour metastasis, inflammation and, possibly, demyelination of neural tissue (reviewed in Yan *et al.*, 1998; Mort and Buttle, 1997; Lah and Kos, 1998).

Regulation of the cathepsin B gene is not well understood. Human cathepsin B pre-mRNA is transcribed from a single copy gene mapping to chromosome 8p22 (Wang *et al.*, 1988). The 5' flanking region of the gene has multiple transcriptional start sites, suggesting more than one promoter (Berquin *et al.*, 1995). The main promoter region is localised 200 bp upstream of exon one (Yan *et al.*, 1998). Whilst this region has features of a housekeeping gene, expression of the cathepsin B mRNA is highly variable between different tissues in the rat (San Segundo *et al.*, 1986) and mouse (Qian *et al.*, 1989). This suggests that expression of the gene may be controlled in a tissue or cell specific manner, and that cathepsin B may participate in tissue specific functions other than intra-endolysosomal protein degradation (San Segundo *et al.*, 1986).

Alternate splicing of the cathepsin B pre-mRNA transcript can result in considerable variation and heterogeneity of the transcripts produced. Most variation between the transcripts is localised in the 5' and 3' untranslated regions. Differences in these untranslated regions may result in different steady state mRNA levels, as well as differing rates of translation (Berquin *et al.*, 1995). Thus regulation of the cathepsin B gene appears to occur at the transcriptional and post-transcriptional level, and may result in considerable differences in the types of transcripts and the level at which they are produced.

The cathepsin B mRNA docks onto the ribosomes of the endoplasmic reticulum (ER) and is transcribed into a pre-propeptide. The signal sequence of the pre-propeptide is cleaved co-translationally and the propeptide folds into its correct conformation within the ER lumen.

Cathepsin B is roughly disk shaped with a diameter of 50 Å and a thickness of 30 Å. The enzyme is bi-lobular with a left (L) and right (R) domain separated by a water-filled interface. The L and R domains interact by polar contacts along this interface. In addition, polypeptide straps starting at the N and C termini, respectively, span the interdomainal interface holding the two lobes together (Musil *et al.*, 1991). Bovine cathepsin B has a further interdomainal strap: a Cys-148→Cys-252 disulfide bridge (amino acid numbering for the mature enzyme) (Baudys *et al.*, 1990).

The interdomainal interface opens to form a V-shaped active site cleft. The active site is buttressed by a unique structural feature, a conformationally flexible 22-residue loop, known as the occluding loop. The propeptide chain runs along the interdomainal interface from the enzyme's N-terminus to the active site. The propeptide is held in place by hydrophobic and hydrophilic contacts along the interdomainal interface. The occluding loop, which normally blocks the rear of the active site, is displaced away from the enzyme surface by the propeptide chain (Turk *et al.*, 1996).

The pro-region of the enzyme prevents the improper activation of the enzyme whilst it is travelling through the biosynthetic and sorting machinery of the cell. In addition, the pro-region increases the stability of the enzyme at alkaline and neutral pH, preventing denaturation (Mort and Buttle, 1997). The enzyme is transferred from the ER to the *cis* face of the Golgi where it is glycosylated with an Asn-linked oligosaccharide. This carbohydrate is phosphorylated by the transfer of a N-acetylglucosaminylphosphate group to the C-6 hydroxyl groups of one or two mannose residues (Hasilik, 1992).

Phosphorylation of the oligosaccharide serves two purposes. Firstly, it prevents the formation of complex oligosaccharides on the enzyme's surface. Secondly it allows the proenzyme to bind to the mannose-6-phosphate receptors (MPRs). These receptors deliver the pro-enzyme to the endolysosomal system, where the acidic conditions trigger the dissociation of the enzymes from their MPRs (Hasilik, 1992). Pro-cathepsin B may also reach the endolysosomal system by MPR-independent systems (Authier *et al.*, 1995; McIntyre *et al.*, 1993; Burge *et al.*, 1991; Hopkins *et al.*, 1990).

Once the enzyme reaches the endolysosomal system, it is activated either autocatalytically or by other proteases. The propeptide region is cleaved off and degraded. Cathepsin B undergoes a further proteolytic cleavage, removing the dipeptide Ala-48/His-49, which

converts the enzyme from a single polypeptide chain into a two chain form. This is the predominant form of the enzyme within the endolysosomal system (Musil *et al.*, 1991).

Several isolation procedures have been described for isolating cathepsin B (Barrett and Kirschke, 1981; Willenbrock and Brocklehurst, 1985a; Rich *et al.*, 1986; Meinesz, 1996). Generally all these methods follow a similar strategy: homogenisation → acid precipitation → ammonium sulfate/acetone fractionation → chromatography. The Sepharose-Gly-Phe-GlySc affinity chromatography method described by Rich *et al.* (1986) was the most effective, with reported yields greater than 100%. Further, the proportion of active enzyme obtained using this method is between 80-100% (Rich *et al.*, 1986). Most other cathepsin B isolates generally contain only about 30-50% active enzyme.

The Gly-Phe-GlySc peptide has a K_i of 63 μM toward cathepsin B, but has a significantly higher affinity for cathepsin L, and does not bind cathepsin H (Rich *et al.*, 1986). Cathepsin L binding to the column is essential irreversible. The different binding affinities of these enzymes towards the ligand allows them to be efficiently separated. Cathepsin B can be eluted from the column using 2,2′-dipyridyl disulfide which forms a thiol-pyridyl link with the active site cysteine of the enzyme, displacing the enzyme from the column. An advantage of this method, compared to other cathepsin B affinity methods, such as aminophenylmercuric chromatography, is that cathepsin B is eluted in an inactive state, preventing autocatalysis. Subsequent treatment of the enzyme-thiol pyridyl derivative with a reducing agent restores the enzyme to full activity.

In the published method (Rich *et al.*, 1986), the affinity matrix was mixed directly with the acetone fraction from the isolation, and then poured into a column. Cathepsin B was subsequently eluted from the column. Two potential problems could arise with this method: firstly, some of the matrix, which is relatively expensive, could be lost when mixing and pouring the matrix-homogenate mixture. Secondly, cathepsin L, with binds to the matrix with great affinity (Rich *et al.*, 1986), is not removed prior to the affinity chromatography step. This could eventually lead to all potential ligand sites in the column being saturated with cathepsin L, with consequent inactivation of the column. Thus modifications to the orginal method as detailed below (Fig. 4.1), were implemented, essentially to remove cathepsin L before the affinity chromatography step.

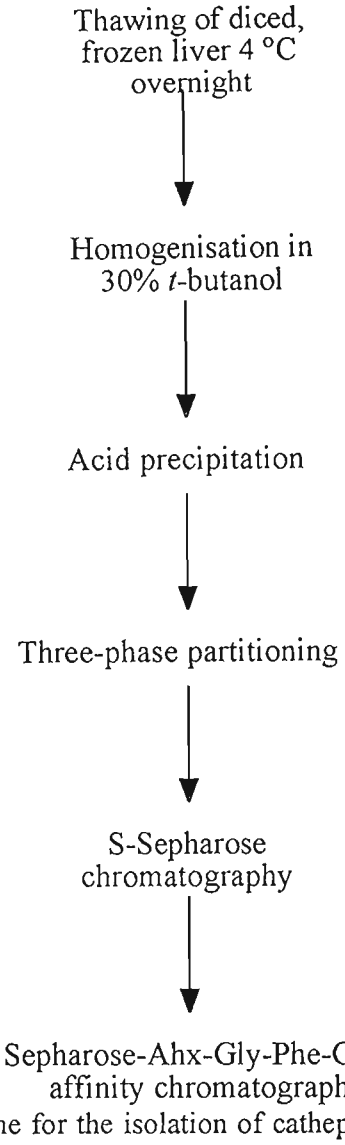

Figure 4.1. Scheme for the isolation of cathepsin B from bovine liver.

The method was also modified 'upstream' of affinity chromatography. *t*-Butanol, at a concentration of *ca.* 30%, was found to inhibit the activity of a variety of proteases, but this activity could be recovered upon removal of the solvent (Dennison *et al.*, 2000). This property allowed enzyme activity in the initial homogenate to be placed in 'suspended animation' allowing greater yields of enzyme in subsequent purification steps (Dennison *et al.*, 2000). It was also discovered (serendipitously!) that such inclusion of tertiary butanol (*t*-butanol) into the homogenisation step of a cathepsin L isolation procedure, reduced the formation of a covalent cathepsin L-stefin B complex. In previous cathepsin L isolation procedures, two cathepsin L fractions could be isolated: a pure cathepsin L fraction, and a covalent cathepsin L-stefin B complex fraction, that was also proteolytically active (Pike *et al.*, 1992). In the original procedure, n-butanol was used to solubilise membranes. However, by including *t*-butanol instead of n-butanol during homogenisation,

cathepsin L-stefin B complex formation was inhibited (Dennison *et al.*, 2000). This suggested that the cathepsin L-stefin B complex was artifactually formed during the homogenisation process; and that including *t*-butanol into the homogenisation step may reduce artifact production.

A further attractive property of including *t*-butanol in the isolation procedure, is that it may reduce interactions between the lysosomal membrane proteins and the lysosomal enzymes themselves. As described in Chapter 2, lysosomal membrane proteins (LAMPs) and hydrolases have the property of aggregating together at low pH. Most cathepsin B isolation procedures include an acid denaturation step, in order to take advantage of the stability of cathepsin B under acidic conditions. However, it has been observed emprically that this step is often associated with a large loss in enzyme yield. This loss in activity may be due to hydrolase-LAMP aggregation. By including *t*-butanol in the isolation procedure, this loss was expected to be minimised. The scheme devised for cathepsin B isolation from bovine liver is shown in Fig. 4.1.

An S-Sepharose cation exchange chromatography step was included before Sepharose-Ahx-Gly-Phe-GlySc chromatography. This was necessary to separate cathepsin B and cathepsin L. Both cathepsins B and L bind to the S-Sepharose matrix, but cathepsin B elutes at a lower salt concentrations than cathepsin L. This prior removal of cathepsin L, prevents irreversible cathepsin L binding to, and thus 'poisoning' of, the Sepharose-Ahx-Gly-Phe-GlySc resin.

4.1.1 Reagents

Homogenisation buffer [150 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, in 50 mM Na-acetate buffer, pH 4.5]. Glacial acetic acid (2.86 ml), NaCl (8.77 g), Na_2EDTA (0.37 g) and NaN_3 (0.2 g) were dissolved in 950 ml dist.H_2O, adjusted to pH 4.5 with NaOH and made up to 1 litre. Before use, *t*-butanol was added to the homogenisation buffer to a final volume of 30%.

Buffer A [1 mM Na_2EDTA, 0.02% NaN_3, in 20 mM Na-acetate buffer, pH 5.0]. Glacial acetic acid (2.29 ml), Na_2EDTA (0.74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 5.0 with NaOH and made up to 2 litres.

Buffer A2 [1 mM Na_2EDTA, 200 mM NaCl, 0.02% NaN_3, in 20 mM Na-acetate buffer, pH 5.0]. Glacial acetic acid (2.29 ml), Na_2EDTA (0.74 g), NaCl (23.38 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 5.0 with NaOH and made up to 2 litres.

Buffer B [50 mM Na-phosphate buffer, pH 6.0, containing 0.02% NaN_3]. $NaH_2PO_4.H_2O$ (7.395 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 1 litre.

Buffer B2 [0.5 M NaCl, 0.02% NaN_3 in 50 mM Na-phosphate buffer, pH 6.0]. $NaH_2PO_4.H_2O$ (0.793 g), NaCl (29.22 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml.

Buffer C [50 mM Na-formate buffer, pH 4.0 containing 0.02% NaN_3]. Formic acid (2.26 ml) and NaN_3 (0.2 g) were dissolved in 900 ml of dist.H_2O, titrated to pH 4.0 with NaOH and made up 1 litre.

S-Sepharose fast flow. The S-Sepharose cation-exchanger was prepared by diluting the supplied hydrated gel 1:2 (v:v) with buffer A, and packing the resulting slurry into a glass column under gravity. The column bed was initially regenerated with 2 M NaCl in buffer A. The same buffer was used to regenerate the column between purification procedures. The column was equilibrated before use by washing with 5 column volumes of Buffer A.

Sepharose-Ahx-Gly-Phe-GlySc affinity resin. This resin was made up as described in Section 3.7. The column was washed with 5 column volumes of Buffer C before use.

Bovine liver. Fresh bovine liver, obtained from the Cato Ridge abattoir, was diced into 2 x 2 cm cubes and frozen at -70°C for at least 3 days, but no longer than a month, before use.

4.1.2 Optimisation of TPP cuts for cathepsin B in bovine liver.

In previous conventional TPP isolation procedures for cathepsin B, an ammonium sulfate cut of between 10-25% was found to give the highest yield of cathepsin B. However, by including 30% *t*-butanol in the homogenisation step, the optimal ammonium sulfate cut for harvesting cathepsin B changed. Several ammonium sulfate cuts were effected on the homogenate and these were analysed for cathepsin B activity.

4.1.2.1 Procedure

Frozen, diced bovine liver was allowed to thaw overnight (12 h) at 4°C to effect release of lysosomal hydrolases from their endocytic compartments (the cell membranes are ruptured by the ice crystals formed during freezing). The liver was homogenised in homogenisation buffer (containing 30% *t*-butanol) at a ratio of 1:2 (liver mass to buffer) in a Waring blendor (max. speed, 3 min/100 g of tissue). This mixture was centrifuged (9000 x g, 20 min, 4°C) to remove any undissolved material. The supernatant was collected and adjusted, with magnetic stirring, to pH 4.2 using glacial acetic acid. This step is important in removing cytosolic inhibitors of the cathepsins. The solution was centrifuged (9000 x g, 20 min, 4°C) to remove acid-precipitated protein, and TPP was effected on the supernatant as described in Section 3.6. An initial ammonium sulfate cut of 10% (m/v) was used, the precipitate collected and dissolved in buffer A (50 ml). Further cuts with 10% (m/v) increments of ammonium sulfate were made to the solution. The precipitates at each step were also collected and dissolved in buffer A (50 ml). The precipitates were dialysed against PEG 20 000 (Section 3.3), followed by buffer A. The solutions had approximately equal volumes after dialysis, and were assayed for protease activity (Section 3.11).

4.1.3 Isolation of bovine cathepsin B.

4.1.3.1 Procedure

Cathepsin B was isolated using a combination of the method of Rich *et al.* (1984), with the modification to the homogenisation steps suggested by Dennison *et al.* (2000). Bovine liver, stored at -70°C, was thawed overnight and homogenised in homogenisation buffer. This mixture was centrifuged, adjusted to pH 4.2, and centrifuged (9000 x g, 20 min, 4°C). Three-phase partitioning, with an ammonium sulfate cut of 20-40% was effected on the supernatant. The precipitates from TPP were dissolved in buffer A, dialysed against PEG 20 000 (Section 3.3), and further dialysed against several changes of buffer A. The resulting dialysate was applied to an S-Sepharose cation-exchanger (2.5 cm x 20 cm). Cathepsin B was eluted using a salt gradient (0-200 mM sodium chloride, i.e. buffer A→buffer A2), that does not elute cathepsin L. Fractions were assayed (Section 3.11), and the cathepsin B fraction from S-Sepharose was dialysed against several changes of Buffer B. DTT was added to the dialysate to a final concentration of 5 mM and the mixture applied to the

Sepharose-Ahx-Gly-Phe-GlySc column. Fluid leaving the column was collected and re-circulated through the column to ensure maximal binding of cathepsin B. The column was washed with two volumes of buffer B2, followed by two volumes of buffer C. Buffer C containing 1.5 mM 2,2′dipyridyl disulfide was loaded onto the column, and left on the column overnight to elute cathepsin B. The eluted fractions were collected, assayed, analysed by electrophoresis, pooled and quantified using the Bradford assay.

4.1.4 Results and discussion

The method chosen to isolate cathepsin B (Fig. 4.1) was based on methods developed in this laboratory and other published isolaton procedures. As described above, the upstream isolation procedure used a novel technique (Dennison *et al.*, 2000), for homogenising bovine liver. By including *t*-butanol in the homogenisation mixture, it was anticipated that artifact formation during homogenisation would be decreased. It was found, however, that the TPP precipitates obtained by this procedure, were infused with *t*-butanol that had to be dialysed out before S-Sepharose chromatography. This meant that the procedure took longer than conventional TPP, but this was off-set by increased yields of cathepsin B (Dennison *et al.*, 2000).

As the precipitates obtained using the new homogenisation process (Dennison *et al.*, 2000) were different from precipitates obtained by conventional TPP, the TPP step had to be optimised. Two independent optimisation procedures were undertaken: both experiments gave identical results, with cathepsin B activity appearing to be greatest in the 20-40% ammonium sulfate range (Fig. 4.2). This range was therefore chosen for the isolation of the enzyme (Section 4.1.3.1).

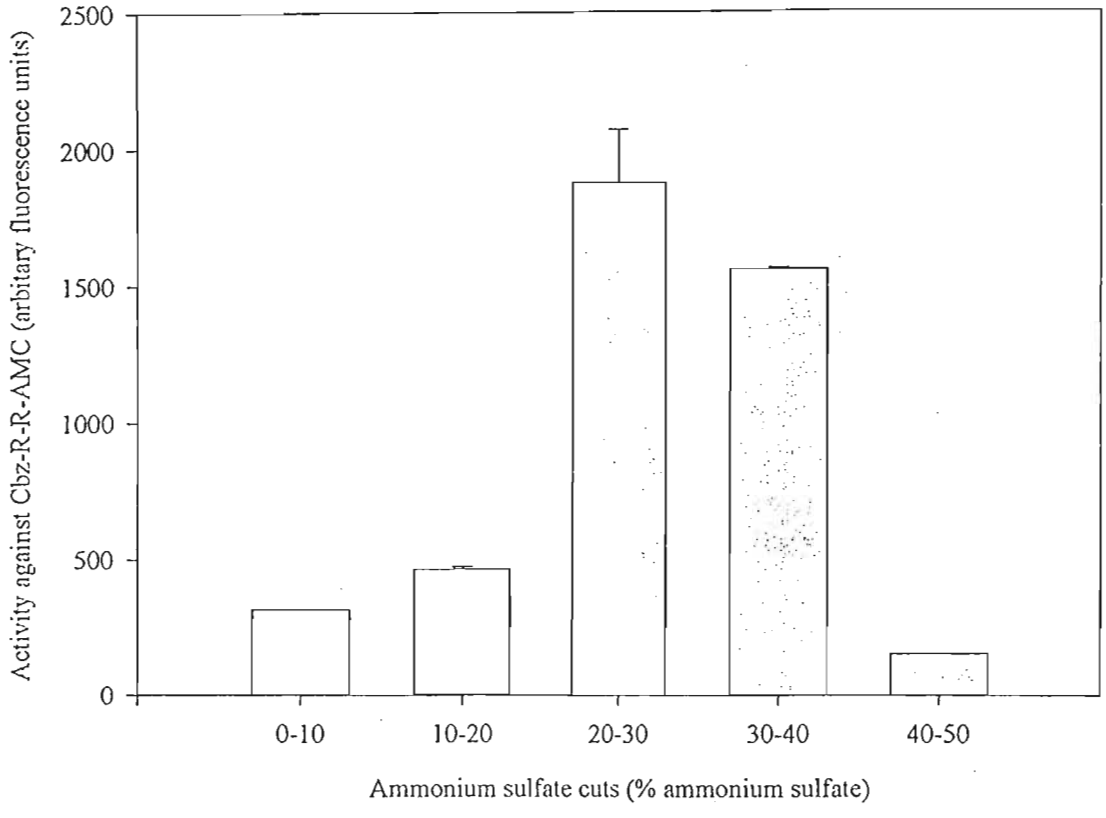

Figure 4.2. Optimal ammonium sulfate cuts for harvesting cathepsin B activity from a liver homogenate via TPP. A bovine liver was homogenised, acid-precipitated and fractionated using 10% ammonium sulfate cuts. The precipitate from each cut was dissolved in equal volumes of buffer A (Section 4.1.1), dialysed to remove excess *t*-butanol, and assayed using Cbz-R-R-AMC (Section 3.11). Each precipitate was assayed in quintiplicate, and the data represents mean ± s.e.

The downstream chromatography procedures were also modified from the original paper by Rich *et al.* (1986). In the present study, cathepsin B and cathepsin L were separated by cation exchange chromatography. Both enzymes bind to cation exchangers with relatively strong affinity. This binding is, in some respects, similar to our proposal that these enzymes may bind to, and aggregate with LAMP proteins (Section 2.6.2). Cathepsin B elutes from an S-Sepharose exchanger within the range, 0-200 mM NaCl, and cathepsin L elutes within the range 200-600 mM NaCl. Thus no cathepsin L should be present at the final Sepharose-Ahx-Gly-Phe-GlySc affinity chromatography step.

Several purifications of cathepsin B were undertaken, sometimes using different bovine livers, or different liver masses. In every case, pure cathepsin B was isolated. The yields of some of these isolations varied from 240% to 15%. These differences in yields may reflect

differences in the starting material, as well as differences in assaying fractions from the various steps. As described above, *t*-butanol decreases enzyme activity, resulting in an apparent loss of activity. The differences in yield may reflect differences in the efficiency of *t*-butanol dialysis from the homogenate and acid precipitate test fractions. Results from one of the isolation procedures are presented in Table 4.1.

Table 4.1 The purification of cathepsin B from bovine liver

	Total protein (mg)	Total activity (μkat)	Specific activity (μkat/mg)	Purification (fold)	Yield (%)
Homogenate	14000	2163	0.155	(1)	(100)
Acid Ppt. supernatant	5657.60	24.22	0.004	0.003	0.011
TPP (20-40%)	312.48	2823	9.03	58.47	130.52
S-Sepharose	57.58	3495	60.71	392.94	161.59
Sepharose-Ahx-Gly-Phe-GlySc	0.738	5208	7057	45680	240.80

The first three steps of the isolation procedure involved crude fractionation steps before chromatography. The purification (fold) at the TPP step is significantly higher than at the preceding homogenisation and acid precipitation steps. Further, the yield appears to increase to 130% (Table 4.1).

These increases do not necessarily reflect efficiency in these isolation steps, but could be partially due to the presence of *t*-butanol in the initial stages of the isolation. Despite attempts at dialysis, and dilution during the assay procedure, the activity of cathepsin B within the homogenate and acid precipitate fractions may have been depressed. This would result in an apparent lower total activity at the start of the purification. This appears to be the case, as the total activity shows an apparent increase during the isolation procedure (Table 4.1) Thus, even the overall yield of 240% may be an exaggeration. In these intial pre-chromatography stages, it was noted that in all isolations undertaken, the total activity of the supernatant at the acid precipitation step was always very low. The basis for this result is not known, as the acid precipitation step, should remove cytosolic cathepsin

inhibitors. These inhibitors should precipitate at low pH, and would therefore be discarded in the acid precipitate. This activity is, however, recovered in latter stages.

Following TPP, the fractions were dialysed to remove excess *t*-butanol, and loaded onto an S-Sepharose column. The chromatogram for this stage of the isolation is shown in Fig. 4.3. In the figure, the 'break-through' peak is not shown as it contains no relevant information. The start of the salt gradient from 0-200 mM NaCl is, however, indicated. Cathepsin B appears to elute in two peaks, which may reflect two different forms of the enzyme, possibly differentially glycosylated.

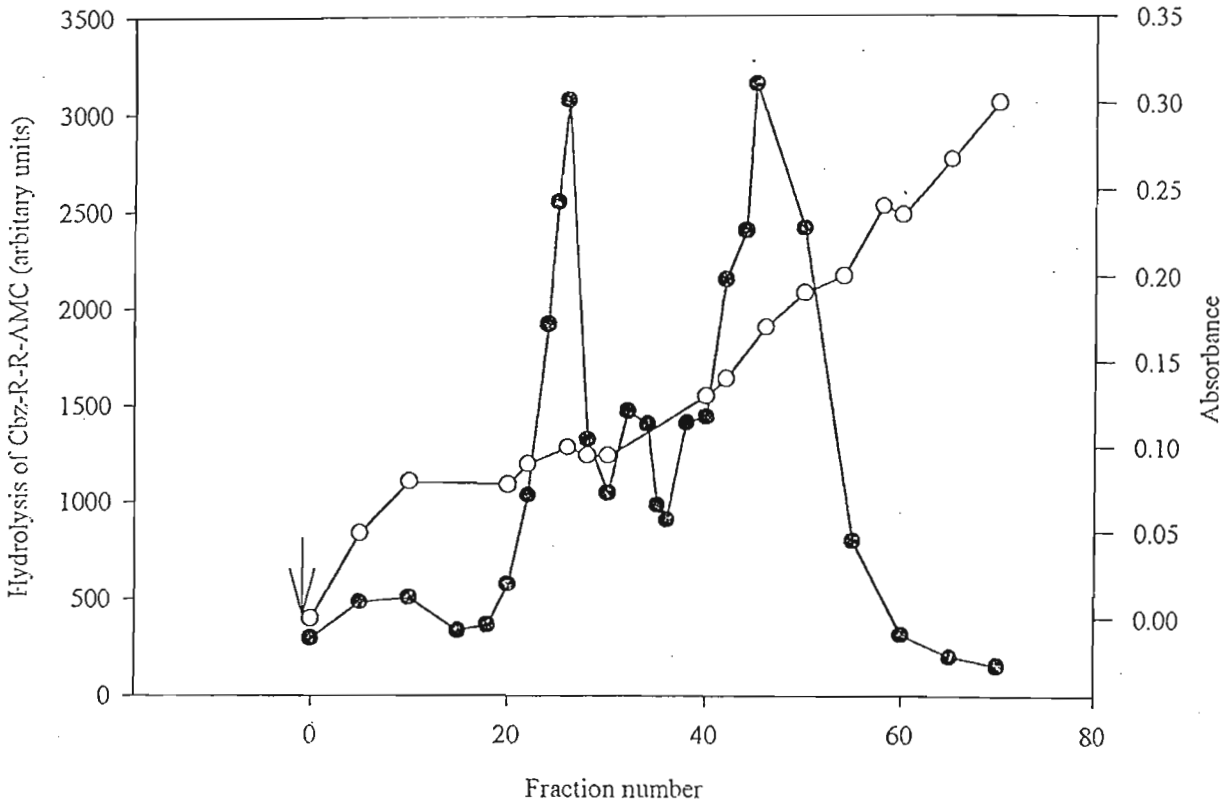

Figure 4.3. Chromatography of the pH 4.2 TPP fraction of cathepsin B on S-Sepharose. The pH 4.2 TPP fraction (Section 4.1.3.1) was loaded onto an S-Sepharose column (2.5 x 17.5 cm; 85.9 ml bed volume), equilibrated in 20 mM Na-acetate, pH 5.0, containing 1 mM Na_2EDTA, 0.02% NaN_3. A 0-200 mM NaCl gradient was applied at ↓ with a flow rate of 10 cm h^{-1} and fractions (8.3 ml) collected. These fractions were analysed for cathepsin B activity (-•-) and A_{280} (-o-).

Active fractions (fractions 20-55) were pooled and analysed for activity and protein concentration. Compared to the TPP step, the yield remained very similar, but the purification increased almost 7 times. This increase could be attributed to the high exchange capacity of the S-Sepharose matrix. As the specific activity of fractions after S-Sepharose chromatography increased significantly, the Sepharose-Ahx-Gly-Phe-GlySc affinity matrix would be expected to bind cathepsin B more efficiently than in the procedures that did not include this step. As described above, cathepsin L is also removed at this step.

The final stage in the isolation procedure is affinity chromatography on Sepharose-Ahx-Gly-Phe-GlySc. The chromatogram for this step shows a single peak of activity and protein content (data not shown). Both purification and yield increase significantly at this step. The specific activity increases 166 fold, reflecting a large increase in purification. The overall yield also increases almost 1.5 times. As most of the *t*-butanol had been dialysed out at this stage, the increase may be attributed to displacement of cytosolic inhibitors from the enzymes. A similar effect was described by Rich *et al.* (1986).

Active fractions from the Sepharose-Ahx-Gly-Phe-GlySc column, were pooled and analysed for activity, electrophoretic purity and for percentage active enzyme. As expected pure cathepsin B was isolated as single (approx. 29 kDa) and two-chain forms (24 and 5 kDa) when run on reducing SDS-PAGE gels (Fig. 4.4). The 5 kDa light chain does not stain very strongly, and is easily distinguished on the gel. A similar result was obtained by Deval *et al.* (1990) who also isolated cathepsin B from bovine liver. E-64 titration of the isolated fractions showed that they were between 85-100% active, a result similar to that achieved by Rich *et al.* (1986).

Using the method described here, cathepsin B could be isolated fairly rapidly, and with good yields. This procedure has two main advantages over the original procedure described by Rich *et al.* (1986). Firstly, the affinity matrix is kept within a column, preventing possible loss of material during the stirring in the original procedure. Secondly, cathepsin L and cathepsin B were separated prior to the affinity step, preventing contamination, and increasing the life of the column. The upstream isolation procedures, homogenisation, acid precipitation and TPP, were novel and have been described in a paper by our group (Dennison *et al.*, 2000). In this procedure enzyme activity within the homogenate is held in 'suspended animation', preventing artifactual reactions and this may have contributed to the very good yields attained.

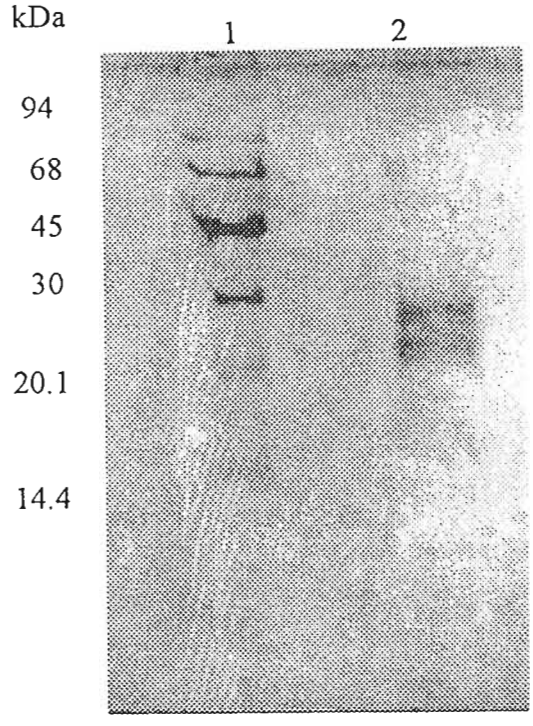

Figure 4.4. Tris-tricine SDS-PAGE of cathepsin B isolated from bovine liver. Cathepsin B fractions from the Sepharose-Ahx-Gly-Phe-GlySc column were boiled in reducing treatment buffer and loaded onto a 10% tricine gel (lane 2). MW markers (phosphorylase b, 94 kDa; BSA 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; lysozyme, 14.4 kDa) are indicated in lane 1.

4.2 Cathepsin D

Cathepsin D [EC 3.4.23.5] is an aspartic protease that is also ubiquitous. Like cathepsin B, its main role within the endolysosomal system is believed to be protein turnover. Cathepsin D is also believed to be responsible for activating the cysteine proteinases, cathepsin B (Van der Stappen *et al.*, 1996) and cathepsin L (Nishimura *et al.*, 1989; Wiederanders and Kirschke, 1989). Cathepsin D is also involved in antigen (Rodriguez and Diment, 1992) and hormone processing (Pillai *et al.*, 1983), and in pathological conditions like tumour metastasis (Capony *et al.*, 1989; Brouillet *et al.*, 1990).

The human cathepsin D gene is expressed in nearly all mammalian tissues. The gene has been localised to chromosome 11 (Hasilik *et al.*, 1982) and is organised into 9 exons and 8 introns (Redecker *et al.*, 1991). The upstream regulatory sequences of the gene lack TATA

and CCAAT boxes, and contain a CpG island and Sp1 binding sites. These features are consistent with those of a housekeeping gene, and are responsible for the nearly ubiquitous expression of the gene (Redecker *et al.*, 1991). Transcription of the cathepsin D gene can be induced by estrogens in MCF-7 (Cavailles *et al.*, 1989) and ZR-75 (Westley and May, 1987) breast cancer cells. Expression of the gene can also be induced in U-937 monocytes by calcitriol, although it is unlikely that calcitriol directly affects the gene (Redecker *et al.*, 1989). Several hormone response elements have been described for the cathepsin D gene and these may be responsible for the regulation of gene expression (Redecker *et al.*, 1991).

Cathepsin D mRNA is translated into a prepropeptide. The signal sequence is cleaved co-translationally and the enzyme folds into its proenzyme conformation within the ER lumen. The propeptide, which prevents improper activation of cathepsin D, is necessary for the enzyme to assume its correct conformation. Cathepsin D deletion mutants lacking the propeptide do not assume the native conformation and are rapidly degraded in a chloroquine-independent fashion (Conner, 1992). Cathepsin D is folded into 3 domains typical of aspartic proteases: an N-terminal domain, a C-terminal domain and an interdomain anti-parallel β-pleated sheet. The interdomain region is made up of the first 7 amino acids of the N-terminal domain (residues 1-7 of the mature human enzyme), the last 16 residues of the C-terminal domain (residues 330-346) and interdomainal linking residues (160-200). The interdomain region forms an active site cleft in which the active site residues Asp-33 and Asp-231 are found, midway. A conformationally flexible β-hairpin structure known as the 'flap' region (residues 72-87), lies above the active site (Baldwin *et al.*, 1993).

Cathepsin D has two N-linked glycosylation sites, one on each domain. Like other lysosomal enzymes, these oligosaccharides are modified by the addition of phosphate monoesters to the mannose residues on the oligosaccharide chain. This modification allows cathepsin D to be transported to the endolysosomal system by the MPR system. Elimination of these glycosylation sites in human fibroblasts results in cathepsin D mutants that are stable, but are not secreted, and are poorly targeted to the lysosome (Fortenberry *et al.*, 1995). In contrast to these results, MPR-independent membrane association and/or transport of cathepsins B and D has been described in rat hepatocytes (Authier *et al.*, 1995) and for procathepsins D and L in macrophages (Diment *et al.*, 1988), Hep G2 cells

(Rijnboutt *et al.*, 1991a; 1991b) and mouse fibroblasts (McIntyre and Erickson, 1991;1993). This discrepancy suggests that MPR-independent transport systems may be cell specific.

Upon delivery to the endolysosomal system, procathepsin D is proteolytically processed to a mature enzyme. In rat, mouse and hamster tissue the enzyme is processed to a single chain form, whilst in human and porcine tissue cathepsin D is ultimately processed to a 2 chain form. Bovine tissue contains equivalent amounts of both forms of the enzyme (Conner *et al.*, 1989). Procathepsin D is processed to a 44 kDa single chain enzyme in a prelysosomal endocytic compartment (Rijnboutt *et al.*, 1992).

The 55 kDa proenzyme can undergo acid-dependent autocatalytic processing to generate pseudocathepsin D *in vitro*. Low pH causes the local denaturation of the propeptide at the active site of the proenzyme. This allows for limited processing of procathepsin D to generate pseudocathepsin D whose size is intermediate between 55 kDa procathepsin D and the 44 kDa single chain enzyme (Conner, 1989). The existence of pseudocathepsin D *in vivo* has been disputed by Richo and Conner (1994). They generated cathepsin D mutants which were incapable of autocatalytic processing *in vitro*, but were still routed to the lysosome and processed to mature enzyme in mouse cells. These results suggest that pseudocathepsin D is not a normal intermediate of cathepsin D processing *in vivo*. In contrast, the gastric aspartic proteinases, pepsinogen, progastricsin and prochymosin appear to be activated by both inter- and intra-molecular proteolytic cleavages (Richter *et al.*, 1998)

In some species, the 44 kDa single chain enzyme is processed into a 2-chain form. The light (15 kDa) and heavy (30 kDa) chains of the 2-chain enzyme are non-covalently associated (Baldwin *et al.*, 1993). The significance of this proteolytic cleavage has not been determined. Cathepsin D processing, from the proenzyme to mature cathepsin D, may be carried out by cysteine proteases. Inhibition of cysteine proteases in cultured cells prevents cathepsin D maturation (Rijnboutt *et al.*, 1991b). Processing of the enzyme begins pre-lysosomally but is completed within the 'lysosome' itself (Rijnboutt *et al.*, 1992).

Most cathepsin D isolations centre on affinity chromatography steps that allow for the rapid isolation of the protease, preventing autolysis and auto-inactivation. Various affinity chromatography ligands have been used for the isolation of cathepsin D including pepstatin (Conner, 1989; Jacobs *et al.*, 1989), a combination of pepstatin and concanavalin A ligands

(Tanji *et al.*, 1991), haemoglobin (Smith and Turk, 1974) and rabbit anti-cathepsin D IgG (Babnik *et al.*, 1984). The isolation used in this study was based on the method of Jacobs *et al.* (1989). This method takes just 6 h and allows for the isolation of cathepsin D from bovine spleen with a yield of *ca.* 8%. This method has essentially two steps: TPP and affinity chromatography on pepstatin-Sepharose. TPP has been described in detail above (Section 3.6). As an established method was used, conventional TPP was employed. An important property of TPP, that is especially relevant in this isolation, is that it denatures haemoglobin, which can be a major contaminant in cathepsin D isolations from spleen and liver tissue (Fortgens, 1996).

The pepstatin-cathepsin D complex is stabilised by hydrogen bond interactions between the main chain atoms of the inhibitor and the side chain and main chain atoms of the enzyme. The central statine residue of the inhibitor is a P1-P1' dipeptide isostere of Leu-Gly in which there is no P1' side-chain substituent. This dipeptide isostere mimics a tetrahedral intermediate in peptide bond hydrolysis by cathepsin D, and is largely responsible for the affinity of cathepsin D for pepstatin (Baldwin *et al.*, 1993; Szewczuk *et al.*, 1992).

The binding of cathepsin D to pepstatin, which depends on hydrogen bond interactions, is reversible and is inefficient at neutral pH values (Knight and Barrett, 1976). By raising the pH, the pepstatin-cathepsin D interaction can be disrupted, allowing the enzyme to be eluted off a pepstatin affinity column.

4.2.1 Reagents

Loading buffer [50 mM Na-acetate, 200 mM NaCl, 0.02% NaN_3, pH 3.5]. Acetic acid (2.86 ml), NaCl (11.69 g) and NaN_3 (0.2 g) were dissolved in 900 ml of dist.H_2O, titrated to pH 3.5 with NaOH and made up to 1 litre.

Elution buffer [50 mM Tris-HCl, 200 mM NaCl, 0.02% NaN_3, pH 8.5]. Tris (6.06 g), NaCl (11.69 g) and NaN_3 (0.2 g) were dissolved in 900 ml of dist.H_2O, titrated to pH 8.5 with HCl and made up to 1 litre.

Bovine liver. Bovine livers were prepared as described in Section 4.1.1.

Pepstatin-diaminohexane-Sepharose. Preparation of this matrix is described in Section 3.8.

4.2.2 Isolation of cathepsin D

Based on the results of previous cathepsin D isolations undertaken in this laboratory (Fortgens, 1996), a TPP cut of 25-35% was used to isolate cathepsin D from bovine liver.

4.2.2.1 Procedure

Bovine liver was allowed to thaw overnight (12 h, 4°C) and homogenised in a Waring blendor (max. speed, 3 min/100 g of tissue) with cold dist.H_2O in a ratio of 1:2 (liver mass to volume of dist.H_2O). This ratio of 1:2 (liver mass to volume of dist.H_2O) was found to give a better recovery of enzyme than a ratio of 1:1 as used by Fortgens (1996). The homogenate was centrifuged (10 000 x g, 30 min, 4°C) to remove any undissolved debris and the supernatant decanted. The pH of the supernatant was adjusted to pH 3.2 using HCl, with constant stirring. Acid precipitated material was removed by centrifugation (10 000 x g, 30 min, 4°C). The supernatant was subjected to TPP (Section 3.6) using an ammonium sulfate cut of 20-35%. The TPP precipitate was dissolved in loading buffer (Section 4.2.1) at approximately one tenth of the volume of the acid precipitate volume. This mixture was centrifuged (15 000 x g, 10 min, 4°C) and filtered through Whatman No. 4 filter paper to remove any undissolved material. The sample was loaded onto a pepstatin-aminohexyl-Sepharose column (1.5 x 0.5 cm = 0.88 ml), that had been previously equilibrated with loading buffer, at 5 cm h^{-1}. The unbound eluate was recirculated through the column overnight to ensure complete binding of cathepsin D to any available pepstatin sites. The column was washed with a further 2 volumes of loading buffer and cathepsin D eluted with elution buffer. Purity of the fractions was determined by SDS-PAGE (Section 3.4) with silver staining (Section 3.5), and the fractions pooled accordingly.

4.2.3 Results and Discussion

Conventional TPP was used to isolate cathepsin D. The precipitates obtained by this method were not infused with *t*-butanol, and hence extensive dialysis steps were not necessary.

Only a single isolation of cathepsin D was undertaken in this study, as this yielded enough cathepsin D for the studies reported in Chapter 5. Unlike the cathepsin B isolation (Table

4.1), the isolation table for cathepsin D has a far more conventional appearance. Yield and total activity appear to decrease, and the purification increases with each step.

Table 4.2 The purification of cathepsin D from bovine liver.

	Total protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	2 480	1577.4	0.636	(1)	(100)
Acid Ppt. supernatant	206.19	940.8	4.562	7.173	59.58
TPP (20-35%)	162.08	445.0	2.746	4.318	28.21
Pepstatin-Sepharose	1.61	51.6	32.050	50.393	3.27

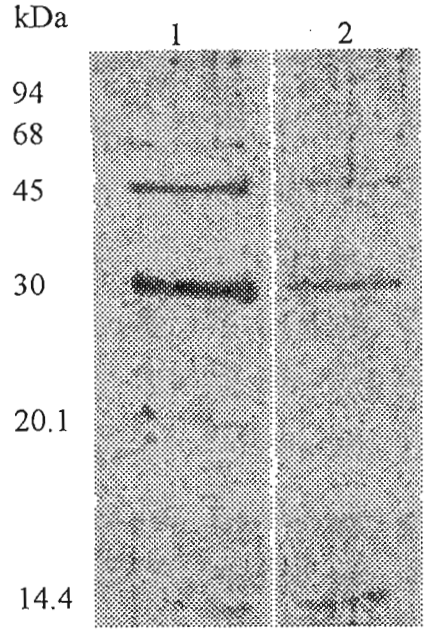

Figure 4.5. Tris-tricine SDS-PAGE of cathepsin D isolated from bovine liver. The pepstatin-Sepharose fraction (lane 2) was boiled in reducing treatment buffer and loaded onto a 10% tricine gel with MW markers (lane 1) (BSA 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; lysozyme, 14.4 kDa).

Pepstatin affinity chromatography gives the highest increase in purificaction. The yield recovered (Table 4.2) was less than that obtained by Jacobs *et al.* (1989) from spleen, but

within a similar range. As expected, cathepsin D was isolated in the single (45 kDa) and two-chain (30 kDa and 15 kDa) forms (Fig. 4.5).

CHAPTER 5

THE REDOX PROPERTIES OF CATHEPSIN B

5.1 Introduction

The nature of the redox environment within the endolysosomal system was reviewed in Chapter 2, although that review was partly informed by the results reported in this chapter. This introduction will focus more on the theory, design and rationale of the experiments undertaken and reported in this chapter. The basic experimental design of assays used in these experiments may be described as follows:

Enzyme + Redox Buffer + Substrate → Result (Activity and Stability)

The activity and stability of cathepsin B, and the activity of cathepsin D were tested using several redox buffers. Both enzymes were isolated using affinity columns, and the enzyme preparations were therefore close to 100% active. This is an important feature when dealing with enzymes sensitive to redox buffers. Redox buffers, especially cysteine-cystine buffers, have the capacity to refold redox-denatured protein (Raman *et al.*, 1996). As described in Chapter 4, conventional cathepsin B isolation procedures produce an enzyme fraction that is between 30-50% active. It has been shown empirically that by increasing the thiol concentration in the assay buffer during E-64 titration (Section 3.1.3), the percentage of active enzyme increases (Coetzer, 1992). In these conventional preparations, it is therefore possible that redox buffers may refold inactive cathepsin B, leading to an increase in the proportion of active enzyme during the assay procedures. By using an affinity matrix, only active enzyme was isolated and used in these assays. Enzyme stocks were periodically checked by E-64 titration to ensure that enzyme had not denatured. In cases where the enzyme had denatured, active enzyme was re-isolated by affinity chromatography.

Conventionally isolated cathepsin B has to be activated by a reducing agent before use, as the enzyme is oxidised during its isolation. Following Sepharose-Ahx-Gly-Phe-GlySc chromatography in the isolation procedure used in this study, cathepsin B is in an inactive enzyme-thiol pyridine form. However, *in vivo*, cathepsin B is already in a reduced, active state (Wilcox and Mason, 1992; Krepela *et al.*, 1997). Thus, cathepsin B was pre-activated

before testing the effect of redox buffers on its activity. Further, the results obtained demonstrate the effect of the redox buffers on the enzyme itself, and not on the ability of the buffers to activate cathepsin B. The redox properties of cathepsin B were tested in actetate-MES-Tris (AMT) buffers. Because of the effect of pH on redox potential (Chapter 2) cathepsin B activity and stability were determined over several pH values. AMT buffers have the capacity to retain a constant ionic strength over a wide pH range (Ellis and Morrison, 1982). This is an important property as it has been shown that cathepsin B activity and stability are influenced by ionic strength (Dehrmann *et al.*, 1996).

The principal lysosomal redox agents are cysteine and cystine (Chapter 2). There are some technical difficulties in working with these reagents. Firstly, cysteine, unlike glutathione (GSH), is rapidly oxidised by molecular oxygen (Mansoor *et al.*, 1992; Newton and Fahey, 1995). This may explain why cysteine is not the principal reducing agent within the cytosol of aerobic cells (Newton and Fahey, 1995). Thus, the buffers have to be deoxygenated before use. Secondly, cystine is poorly soluble in aqueous solutions. This difficulty was overcome, when making up AMT buffers containing cystine, by adding the cystine after the addition of acetic acid to water.

The effect of redox potential, and differing concentrations and ratios of cysteine and cystine on enzyme activity was tested. If there were changes in the activity of the enzyme, these changes would be quantified both kinetically and thermodynamically. The kinetic changes would have been monitored using conventional kinetic parameters: the Michaelis constant (K_m), maximum velocity (V_{max}), and the turnover number (k_{cat}). The thermodynamic parameters would have been described in terms of the equilibrium constant (K_{eq}) of the reaction:

Cathepsin B-Cystine (inactive) + 2 Cysteine $\leftrightarrow$ Cathepsin B (active) + Cystine

The K_{eq} of the reaction would reflect how stable the disulfide bond between (oxidised, inactive) cathepsin B and cystine is, compared to cysteine. If the K_{eq} is of similar magnitude to the *in vivo* ratio of cystine/cysteine, then redox regulation of the enzyme is possible. The thiol/disulfide ratios within the endolysosomal system are not known, but an estimate of the thiol concentration has been given by Krepela *et al.* (1999). If on the other hand, there are no changes in the activity of the enzyme with redox potential, then the enzyme may not be redox regulated.

The enzyme's half-life ($t_{1/2}$) gives a measure of its stability. What is actually measured, is the time it takes for the activity of the enzyme to decrease by half. The theory of how half-life is determined is given by Dennison *et al.* (1992). Basically, it is assumed that loss of activity is a first-order process and can be monitored by measuring the loss of activity over a period. The rate constant for this loss of activity (k_{obs}) can be determined from activity versus time curves, and the stability determined by the following equation (Segal, 1976):

$$t_{1/2} = 0.693/k_{obs} \qquad (2)$$

For this measure to be valid, it is important that substrate is never limiting, and that there is a measurable loss of activity over the time period employed. It was sometimes necessary to alter the enzyme concentration to meet these conditions.

Table 5.1 Properties of whole protein substrates used in activity assays.

Name	Molecular weight (kDa)	Subunits	No. of disulfide bridges	No. of free cysteine(s)	Metal moieties
Haemoglobin	62	4	0	2	4
BSA	69.29	1	17	1	0
RA-BSA	~70	1	0	0	0
Lysozyme	16.29	1	4	1	0

Most of the assays described in this chapter were undertaken using a fluorogenic substrate. Whole protein assays were also included in the light of several reports that have described the effect of reducing agents on the processing and/or antigenic presentation of whole protein substrates (Kooistra *et al.* 1982; Collins *et al.*, 1991; and Merkel *et al.*, 1995). Four substrates, haemoglobin, bovine serum albumin (BSA), reduced and alkylated BSA (RA-BSA) and chicken egg lysosome, were tested. These substrates were chosen because of their differing structural properties (Table 5.1), with the hope that the effect of redox buffers on proteolysis could be determined.

5.2 Reagents

Oxygen-free nitrogen. Oxygen-free nitrogen was from Afrox Ltd. (South Africa).

Acetate-MES-Tris buffers [final concentration in assay = 100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA]. AMT buffers retain a constant ionic strength over a wide pH range (Ellis and Morrison, 1982), and were used to construct redox buffers. All buffer solutions were deoxygenated by boiling, cooling under a stream of oxygen-free nitrogen, and degassed under vacuum. To these buffers was added an appropriate concentration of reducing agent to accommodate dilution during the assays employed. For those buffers containing cystine, the disulfide was added to the stock AMT buffers. Further steps were done under a constant stream of oxygen-free nitrogen. Using the method of Gaitonde (1967) (Section 3.10) it was shown that the thiol concentration within the buffers did not change significantly over the time periods employed in these experiments.

Unfolding buffer [50 mM Tris-acetic acid, 8 M urea, 0.02% NaN_3 pH 8]. Tris (1.51 g), urea (120.12 g) and NaN_3 (0.05 g) were dissolved in 100 ml of ddH_2O, titrated to pH 8 with acetic acid and made up to a final volume of 250 ml.

1 mM Ammonia. 68 µl of a 25% ammonia solution was made to 1 litre with ddH_2O.

Storage solution. NaN_3 (0.01 g) was dissolved in ddH_2O (50 ml).

Sep-pak C18 thiol elution buffer [35% acetonitrile, 0.01% trifluoroacetic acid]. Acetonitrile (35 ml) and trifluoroacetic acid (10 µl) were added to ddH_2O and made up to a final volume of 100 ml.

Sep-pak C18 protein elution buffer [65% acetonitrile, 0.01% trifluoroacetic acid]. Acetonitrile (65 ml) and trifluoroacetic acid (10 µl) were added to ddH_2O and made up to a final volume of 100 ml.

Reduced/alkylated BSA (2 mg/ml). Reduced-alkylated BSA (RA-BSA) was prepared using the method of Mego (1984). BSA (0.04 g) was mixed with a 10-fold molar excess (with respect to disulfide bridges) of DTT (0.0152 g) in unfolding buffer (4 h, RT). A two-fold molar excess (with respect to DTT) of iodoacetate (0.0364 g) was added to the solution, which was left at room temperature for 30 min. The protein solution was dialysed against

several changes of a 1 mM ammonia solution and further dialysed against storage solution, followed by PEG 20 000, and adjusted to give a final concentration of 2 mg/ml.

To determine whether the RA-BSA substrate had been completely reduced and alkylated, a novel method based on those of Wynn and Richards (1995) and Habeeb (1975), was developed. RA-BSA (5 ml, 2 mg/ml) was reduced with DTT (10 mM, 30 min, RT) and the resulting mixture was acidified to pH 3 with HCl. The mixture was injected into a Sep-pak C18 column pre-wet with Sep-pak C18 thiol elution buffer. The column was rinsed with this buffer to elute the thiol, and fractions (1 ml) collected. These fractions were tested for the presence of thiol using the DTNB reagent (Section 3.10). Once thiol could no longer be detected, the column was washed with Sep-pak C18 protein elution buffer, to elute the protein. The protein fractions were collected and quantified at 280 nm. The presence of free thiol on the proteins was determined using the method of Habeeb (1975) (Section 3.10). Using this method it was shown that the thiol moieties on the reduced RA-BSA, unlike those on native BSA, had all been alkylated.

Protein substrates (2 mg/ml). The proteins, haemoglobin, BSA, and lysozyme were separately dissolved in storage solution at 2 mg/ml.

Cathepsin B assay buffer 2 [0.1 M sodium phosphate, 4 mM EDTA, 2 mM DTT, pH 6]. NaH_2PO_4 (6.90 g) and Na_2EDTA (0.93 g) were dissolved in 450 ml dist.H_2O, adjusted to pH 6.0 with NaOH and made up to 500 ml. Immediately before use, dithiothreitol was added to 5 mM (i.e. 0.204 g/500 ml).

Cathepsin D assay buffer 2 [100 mM sodium citrate, 0.02% NaN_3, pH 3.2]. Citric acid (10.52 g) and NaN_3 (0.1 g) were dissolved in about 450 ml of dist.H_2O, adjusted to pH 3.2 with NaOH and made up to 500 ml.

5% TCA. TCA (5 g) was dissolved in ddH_20 (60 ml), and made up to a final volume of 100 ml.

Cathepsin B and cathepsin D. The isolation of these proteases is described in Chapter 4.

5.3 Experimental Procedures

5.3.1 The effect of pH and various thiol concentrations on cathepsin B activity and stability.

Cathepsin B was pre-activated in cathepsin B assay buffer 2 (Section 5.2) for 10 min, 25°C. The enzyme (250 ng, 32 µl) was added to pre-warmed AMT buffer (pH 5-7, 843 µl) containing an appropriate concentration of thiol (0-20 mM), and Cbz-R-R-AMC (125 µl, 100 µM). The reaction was monitored continuously at 37°C in a Hitachi F-2000 spectrofluorometer. Experiments to determine the half-life of cathepsin B under these redox conditions were conducted as described previously (Dennison *et al.*, 1992; Dehrmann *et al.*, 1995). The enzyme concentration was adjusted in these experiments to ensure that there was a measurable loss in enzyme activity over the assay periods employed (< 15 min) (Dennison *et al.*, 1992). Longer assay times resulted in appreciable oxidation of the redox buffers, and were therefore not used. The data represents the mean ± standard error of at least 3 independent determinations.

5.3.2 The effect of cystine on cathepsin B activity and stability.

Cathepsin B was pre-activated in cathepsin B assay buffer 2 (10 min, 25°C). The activated enzyme (250 ng, 32 µl) was added to pre-warmed AMT buffers with or without cystine (0-0.8 mM, 843 µl), containing an appropriate concentration of thiol (0-20 mM), and Cbz-R-R-AMC (125 µl, 100 µM). These assays were undertaken in triplicate and the mean and standard error were determined. The reactions were monitored continuously at 37°C in a Hitachi F-2000 spectrofluorometer. In order to determine the effect of cystine on the stability of cathepsin B, half-life assays were conducted at pH 7.0 in AMT buffers with varying concentrations of cystine (0-0.8 mM) and cysteine (0-20 mM). At least 2 independent experiments were conducted from which the mean and standard errors were calculated.

5.3.3 The effect of redox conditions on cathepsin hydrolysis of protein substrates.

Cathepsin B was pre-activated in cathepsin B assay buffer 2 (2 min, 37°C). The activated enzyme (1 µg, 88 µl) was added to pre-warmed AMT buffer with or without cystine (0-0.8 mM, 787 µl) containing an appropriate concentration of thiol (0-10 mM), and protein

substrate (125 µl, 2 mg/ml). An aliquot (300 µl) was immediately removed and added to 5% TCA (240 µl) as a zero time control. After 10 min at 37°C, a further aliquot (300 µl) (was removed) and added to 5% TCA (240 µl). The reaction mixes were centrifuged and the absorbance quantified at 280 nm.

For the cathepsin D assays, the enzyme was pre-warmed in cathepsin D assay buffer 2 (Section 5.2) for 2 min at 37°C. Warmed enzyme (4 µg) was added to pre-warmed assay buffer (750 µl) and protein substrate (125 µl, 2mg/ml). An aliquot (300 µl) was immediately removed and added to 5% TCA (240 µl). After 10 min at 37°C, a further aliquot (300 µl) was removed and added to 5% TCA (240 µl). The reaction mixes were centrifuged and the absorbance quantified at 280 nm. At least 3 independent experiments were conducted from which the mean and standard errors were calculated. Control experiments (assays without enzyme) were also undertaken to determine whether the susbstrates were spontaneously denaturing/hydrolysing within the redox buffers themselves. No such effect was found.

5.3.4 Calculation of redox potential for redox buffers.

Refer to Appendix 1

5.4 Results

5.4.1 The effect of pH and thiol concentration on cathepsin B activity.

The effect of thiol concentration (provided by cysteine or glutathione) and pH on cathepsin B activity against Cbz-R-R-AMC was explored using AMT buffers of constant ionic strength, with varying concentrations of thiol. In contrast to other reports (see for example Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992; Dehrmann *et al.*, 1996), the activity of cathepsin B at pH 6.0 and pH 7.0, over all thiol concentrations, was similar; and was greater than at pH 5.0 (Fig. 5.1). Despite GSH having a lower standard reduction potential than cysteine (Segal, 1976), a GSH-containing buffer of pH 7.0, gave significantly lower enzyme activity over the range of thiol concentrations tested, compared to cysteine-containing AMT buffers at pH 7.0 (Fig. 5.1).

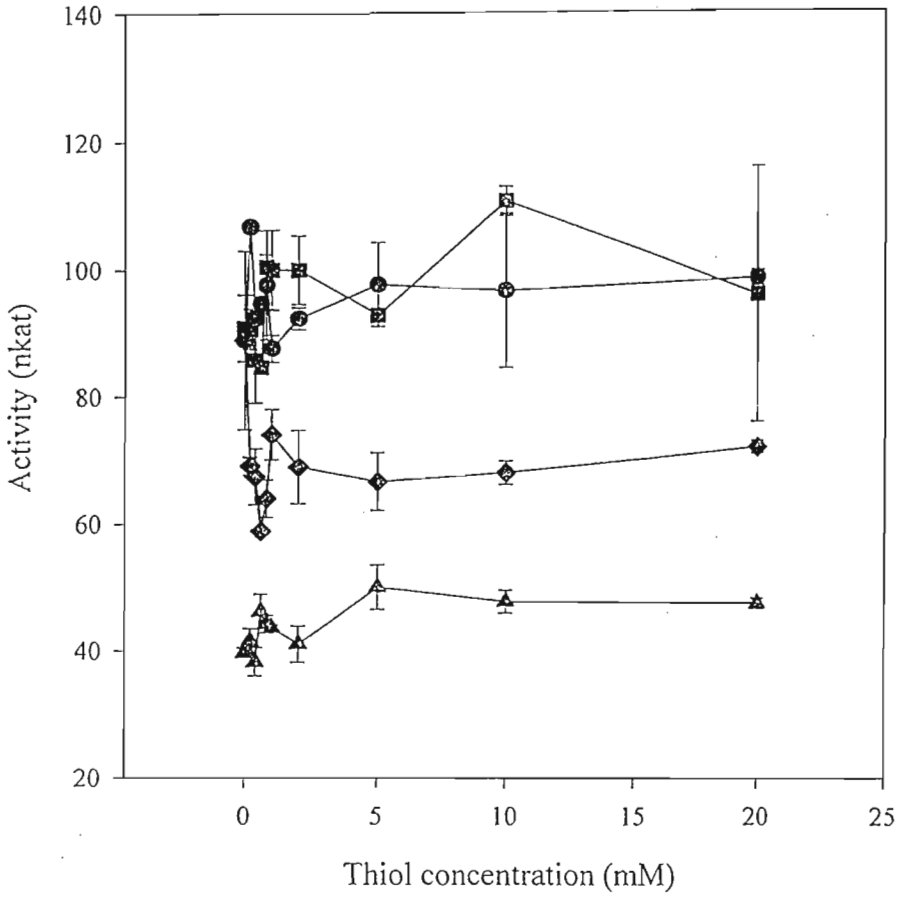

Figure 5.1. The effect of thiol concentration on cathepsin B activity. The effect of thiol concentration on cathepsin B activity against Cbz-R-R-AMC was monitored in AMT redox buffers containing cysteine containing at pH 7 (■), pH 6 (●), pH 5 (▲), and in GSH-containing buffers at pH 7 (◆). The reactions were monitored continuously at 37°C, and the data represent the mean ± standard error of at least 3 independent determinations.

The effect of cysteine on the half-life of cathepsin B at pH 6.0 and 7.0 is shown in Fig. 5.2 (the enzyme was too stable at pH 5 for a half-life to be measured). In contrast to the activity (Fig. 5.1), the half-life was significantly greater at pH 6.0 than at pH 7.0. The stability of cathepsin B at pH 7.0, in the absence of thiol (AMT redox buffer, pH 7.0, no cysteine), was generally significantly lower than in the presence of thiol (AMT redox buffers, pH 7.0, 0.2-20 mM cysteine) (Fig. 5.3a).

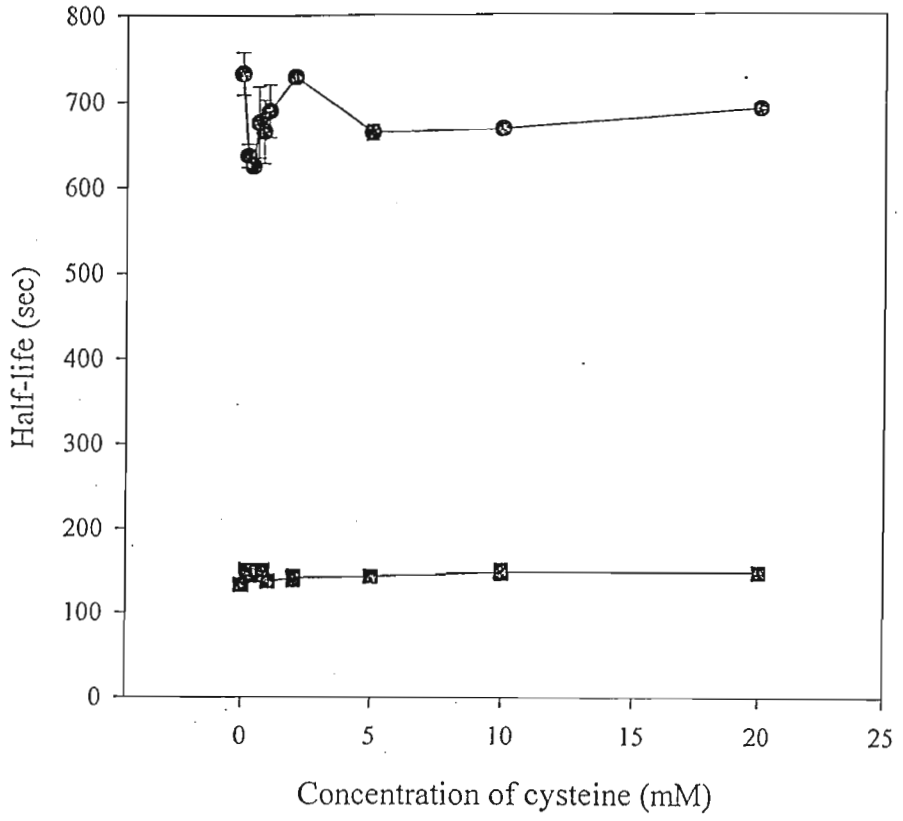

Figure 5.2. The effect of thiol concentration on cathepsin B stability. The effect of thiol concentration on cathepsin B stability was monitored in cysteine-containing AMT redox buffers at pH 7 (■) and pH 6 (●). The reactions were monitored continuously at 37°C, and the data represent the mean ± standard error of at least 3 independent determinations.

It appears that cysteine alone increases the stability of cathepsin B at pH 7.0. This is consistent with the result of Dehrmann *et al.* (1996). In contrast, at pH 6.0, cysteine apparently has little effect, as the enzyme was as stable with as without cysteine (Fig. 5.3b). Krepela *et al.* (1999) reported that with increases in cysteine concentration, cathepsin B showed a local minimum in activity. These authors believed that this effect was due to cysteine thiol inhibition of the enzyme. Data from this study also shows this effect over all pHs tested, and with GSH-containing buffers (Fig. 5.1). Further, the present results of the effect of cysteine on cathepsin B stability (Fig. 5.2) also showed local minima with increases in thiol concentration. These minima corresponded to the minima in the activity data (Fig. 5.3), suggesting a common mechanism that affects both stability and activity.

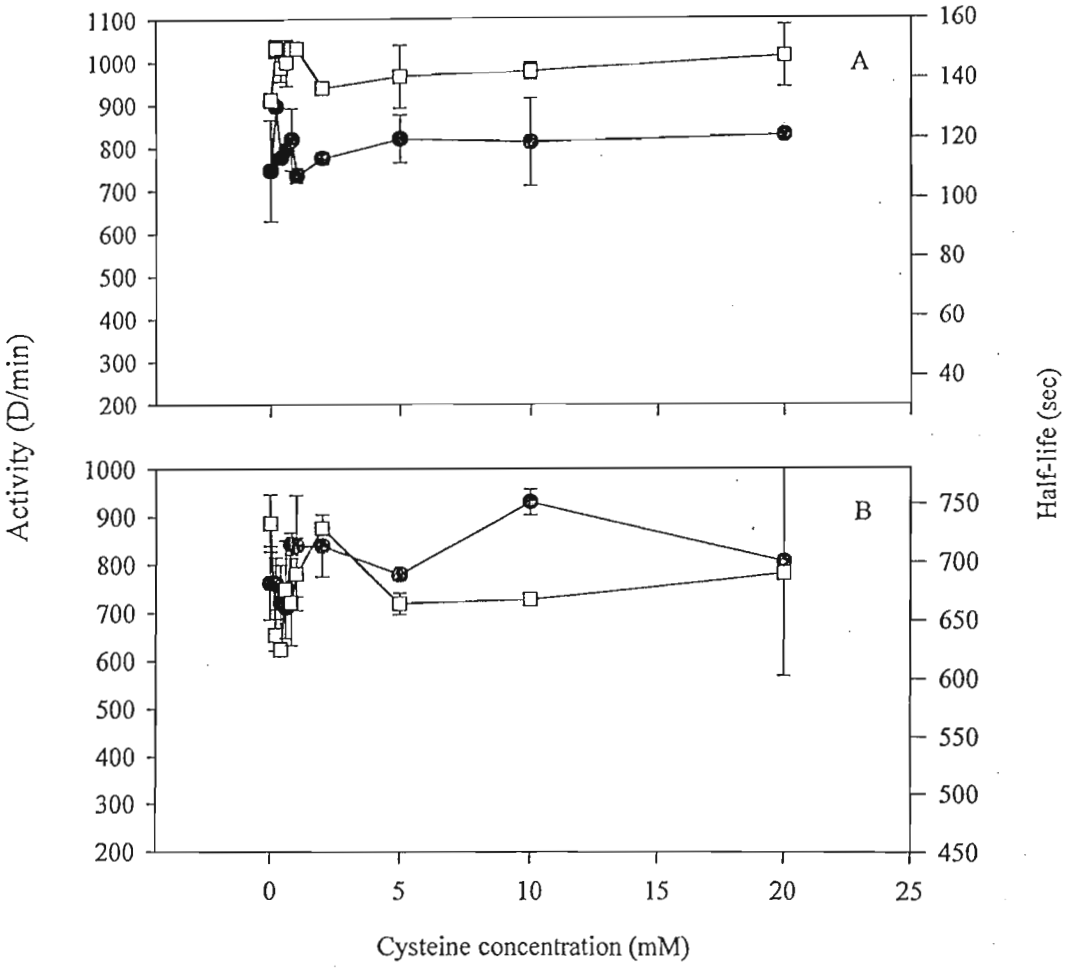

Figure 5.3. A comparison of the effect of cysteine on cathepsin B stability and activity at pH 7.0 (A) and pH 6.0 (B). The data describing the effect of cysteine on cathepsin B activity (●) and stability (□) in AMT redox buffers was compared. The data represent the mean and standard errors of least 3 independent determinations.

5.4.2 The effect of cystine on cathepsin B activity and stability.

Most assays of cysteine protease activity do not include cystine. However, adding cystine to the buffers enabled calculation of reduction potentials using the Nernst equation. At pH 6.0, starting in a buffer without cysteine, cathepsin B activity increased with decreasing reduction potential, until approximately -80 mV, and thereafter was insensitive to changes in reduction potential (Fig. 5.4a). This suggests that at pH 6.0, cathepsin B is capable of

operating over a broad cysteine/cystine generated redox potential range of -80 mV to -350 mV. Similarly, at pH 7.0, the concentration of cystine does not appear to have a significant effect on the *activity* of cathepsin B (Fig. 5.4b).

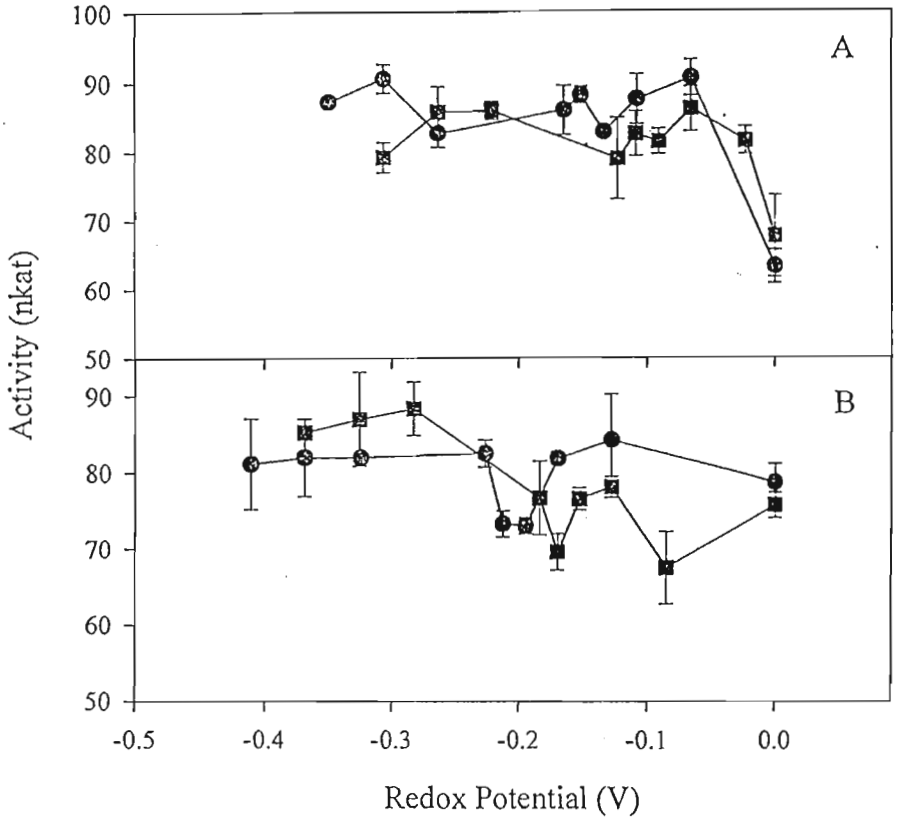

Figure 5.4. The effect of redox potential and cystine concentration on cathepsin B activity at pH 6 (A) and pH 7 (B). Cathepsin B activity against Cbz-R-R-AMC was assessed with various concentrations of cysteine (0-20 mM) in AMT redox buffers containing cystine at concentrations of 0.2 mM (●) or 0.8 mM (■) respectively. The redox potential of each of the buffers was calculated using the Nernst equation. The data points at 0 mV represents an AMT redox buffer with cystine (0.2-0.8 mM), but no cysteine. The reactions were monitored continuously at 37°C, and the data represent the mean ± standard error of at least 3 independent determinations.

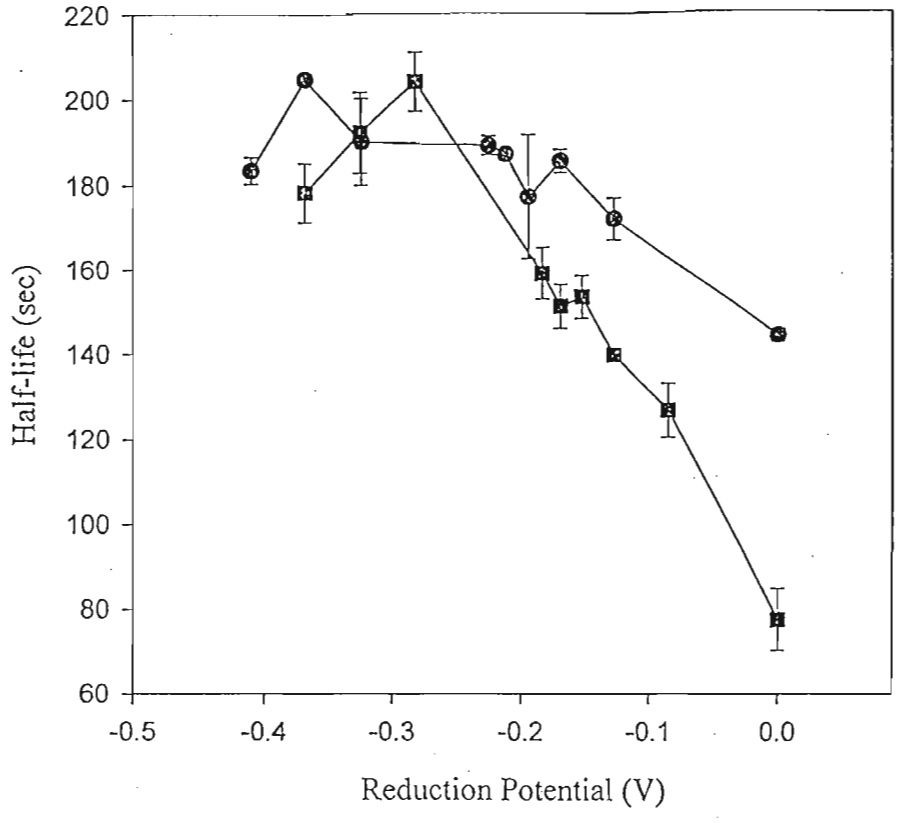

Figure 5.5. The effect of redox potential and cystine concentration on cathepsin B stability at pH 7. Cathepsin B stability against Cbz-R-R-AMC was assessed in AMT redox buffers containing various concentrations of cysteine (0-20 mM), and 0.2 mM cystine (●) or 0.8 mM cystine (■). The redox potential of each of the buffers was calculated using the Nernst equation. The data points at 0 mV represents an AMT redox buffer with cystine (0.2-0.8 mM), but no cysteine. The data represent the mean ± standard error of at least 2 independent determinations.

As described, assays were of limited duration to prevent oxidation of redox buffers. The inherent stability of cathepsin B at pH 6.0 made it difficult to assess the effect of cystine on stability at this pH. At pH 7.0 on the other hand, cystine had a marked effect on the *stability* of cathepsin B. Between buffers with no cysteine and those with reduction potentials of -250 mV, a linear relationship between stability and reduction potential was found (Fig. 5.5), with enzyme *stability* increasing with decreasing redox potential (i.e. increasing concentrations of cysteine). For a given redox potential, stability was reduced in buffers containing 0.8 mM cystine compared to buffers with 0.2 mM cystine.

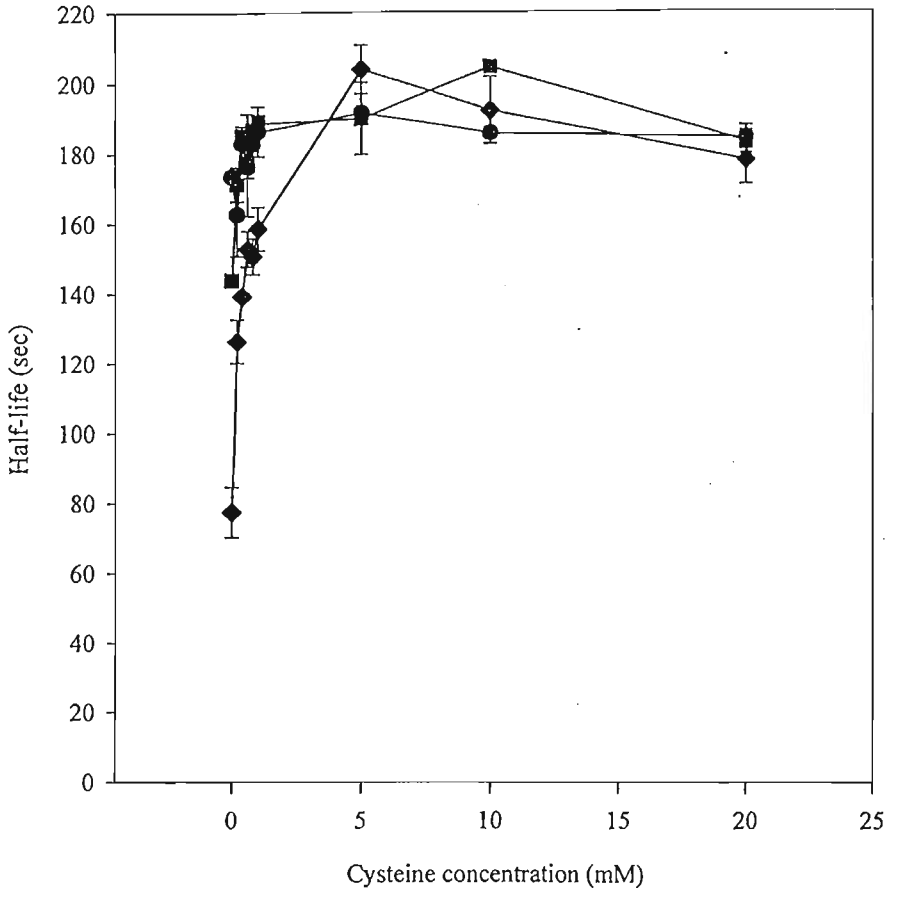

Figure 5.6. A comparison of the effect of cystine on cathepsin B stability at pH 7.0. Cathepsin B stability was measures in AMT redox buffers containing cysteine (0-20 mM) and cystine at 0 mM (●), 0.2 mM (■) and 0.8 mM (♦). The data represent the mean and standard error of at least 2 independent experiments.

This effect appeared to be dose dependent with stability greater in buffers with no cystine, followed by buffers with 0.2 mM cystine, and then buffers with 0.8 mM cystine (Fig. 5.6). However, below -250 mV, with decreasing redox potential (i.e. increasing cysteine concentration), the effect of cystine was ameliorated (Fig. 5.5). These results are consistent with cathepsin B undergoing cystine-dependent oxidation, resulting in decreased stability (Fig. 5.5), but in a manner that apparently did not affect the enzyme's activity (Fig. 5.4b).

5.4.3 The effect of redox buffers on cathepsin B hydrolysis of whole protein substrates

Haemoglobin, BSA, RA-BSA and lysosome were selected as substrates because of their distinct structural features. Haemoglobin, is a tetramer with no disulfide bridges and two free cysteines; BSA has seventeen disulfide bridges, and one free cysteine; RA-BSA has no free thiols or disulfide bridges; and lysozyme has four disulfide bridges and a free cysteine. The last three proteins are monomeric (Table 5.1).

With increasing concentration of cysteine, cathepsin B activity against haemoglobin showed no consistent trend but instead a local minimum at about 0.5→1 mM, and local maxima at 0- and 5 mM cysteine, in both the presence or absence of cystine (Fig. 5.7a) were evident. Cystine, at 0.8 mM, appeared to increase the rate compared to rates with 0 or 0.2 mM cystine, over the entire tested range of 0→10 mM cysteine. This stimulatory effect was not linear, however, as rates with 0 mM cystine were, at some cysteine concentrations, greater than rates with 0.2 mM cystine and at others were less than with 0.2 mM cystine. However, at either high or low concentrations of cysteine, cystine appeared to increase the rates of haemoglobin hydrolysis.

Cystine exerted a similar effect on the hydrolysis of RA-BSA by cathepsin B (Fig. 5.7b). At lower thiol concentrations, in the presence of differing concentrations of cystine, the rates of hydrolysis were approximately similar (Fig. 5.7b). However, at higher thiol concentrations (> 1 mM), cystine appeares to increase the rates of cathepsin B hydrolysis of RA-BSA. This effect was greatest with 0.8 mM cystine. Also conspicuous, was that the activity of cathepsin B against RA-BSA, like that for haemoglobin, was relatively high in the absence of thiol, and in the presence of 0.8 mM cystine, when compared to buffers with no cystine or 0.2 mM cystine (Fig. 5.7a-b). The activity at 0.8 mM cystine was also high at relatively high concentrations of cysteine.

It is likely that cystine exerts this effect on hydrolysis, only in absence of disulfide links in the substrate. Both BSA and lysosome did not show uniform increases in the hydrolysis rates, with increases in cystine concentration (Fig. 5.7c-d). In the case of BSA, cystine appeared to exert no significant effect on cathepsin B activity against the substrate. At low thiol concentrations (< 1 mM), lysozyme hydrolysis by cathepsin B occurred at similar rates in the presence of different concentrations of cystine. However, at 5 mM cysteine,

the hydrolysis of lysozyme occurred at a greater rate in the presence of 0.2 mM cystine. In contrast, the activity of cathepsin B at 5 mM cysteine, and in the presence of 0.8 mM cystine, was depressed when compared to the experiments without cystine (Fig. 5.7d). In the case of both BSA and lysozyme, the local minimum effect in activity, with increasing cysteine concentration (Fig. 5.3) was not present. The rates of hydrolysis of all the protein substrates tested, showed no relationship to redox potential (data not shown).

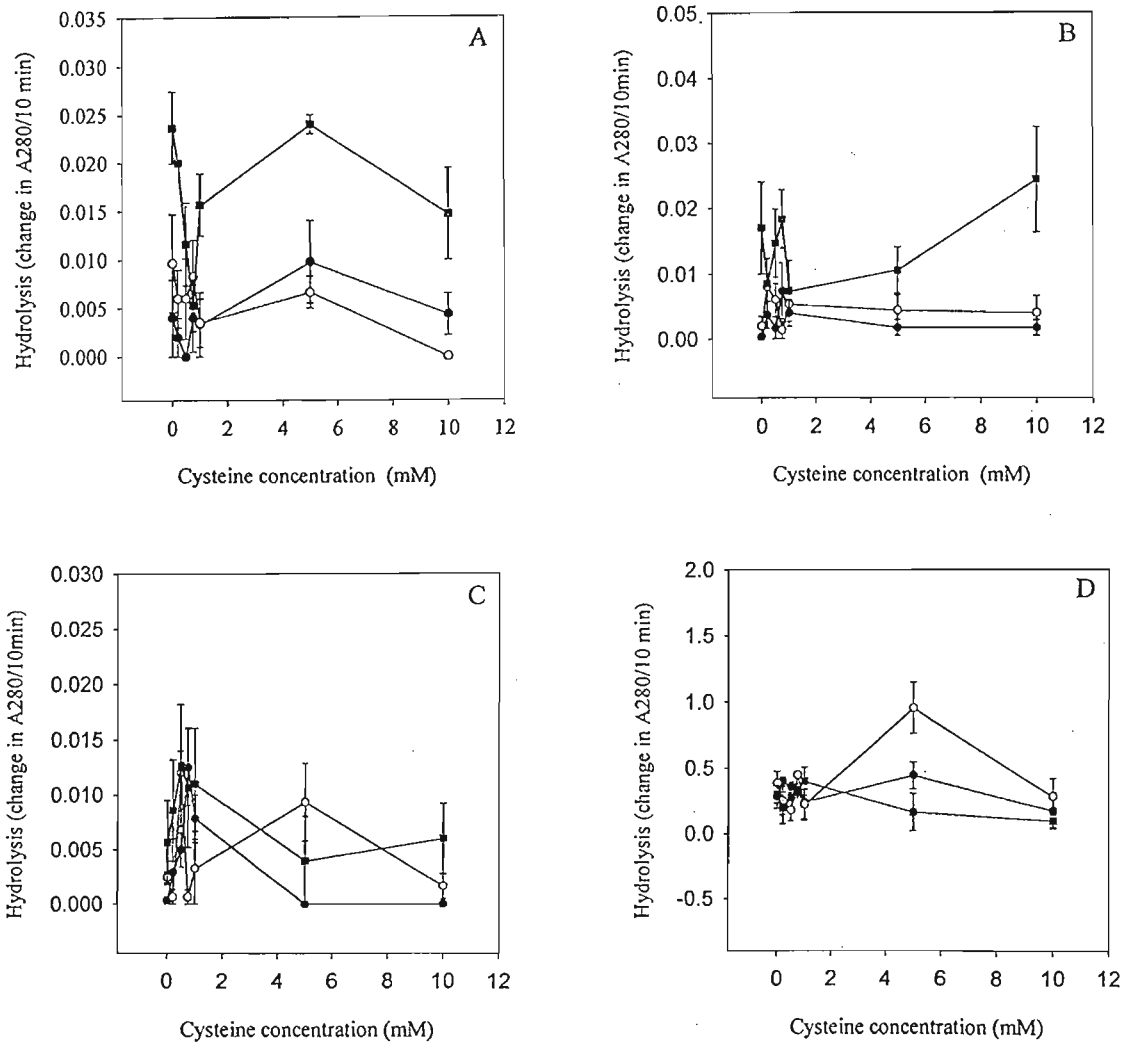

Figure 5.7. The effect of cysteine:cystine AMT redox buffers on cathepsin B hydrolysis of haemoglobin (A), RA-BSA (B), BSA (C) and lysozyme (D) was assessed at pH 6.0. The redox buffers contained cysteine (0-10 mM) and cystine at 0 mM (○), 0.2 mM (●) and 0.8 mM (■). The data represent the mean ± standard error of 3 independent experiments.

The effect of cystine on cathepsin B activity against whole protein substrates is enigmatic. The activity against substrates with no disulfide bonds appears to be stimulated by the presence of cystine but the activity against substrates with disulfide bonds was not stimulated. The activity of cathepsin B against the fluorometric substrates indicated that cystine did not significantly increase activity (Fig. 5.4), and may actually contribute to oxidative denaturation of the enzyme (Fig. 5.5). Further, control experiments demonstrated that the redox buffers themselves were not denaturing the substrate. This suggests that the stimulatory effect that cystine exerts on cathepsin B hydrolysis of substrates without disulfide bridges, may be related to interaction of the enzyme with substrate. To determine whether this effect is a general feature of lysosomal proteolysis, the action of cathepsin D against these substrates was assessed.

5.4.4 The effect of redox buffers on cathepsin D hydrolysis of whole protein substrates

Cathepsin D is a lysosomal aspartic protease which operates at pH values less than pH 4.0. At this low pH thiol-disulfide exchange would be inhibited. Cathepsin D hydrolysis of haemoglobin (Fig. 5.8a) showed that the presence of 0.8 mM cystine did not significantly affect proteolysis at low cysteine concentrations (< 1 mM). However, at thiol concentrations greater than 1 mM the hydrolysis of haemoglobin was stimulated by the presence of cystine. A similar pattern of hydrolysis was observed for cathepsin D hydrolysis of RA-BSA (Fig. 5.8b), with the highest rates of hydrolysis noted with 0.8 mM cystine and 10 mM cysteine. In contrast, cystine did not appear to stimulate BSA hydrolysis by cathepsin D (Fig. 5.8c). On the other hand, cystine appeared to stimulate lysozyme hydrolysis by cathepsin D at thiol concentrations greater than 1 mM (Fig. 5.8d).

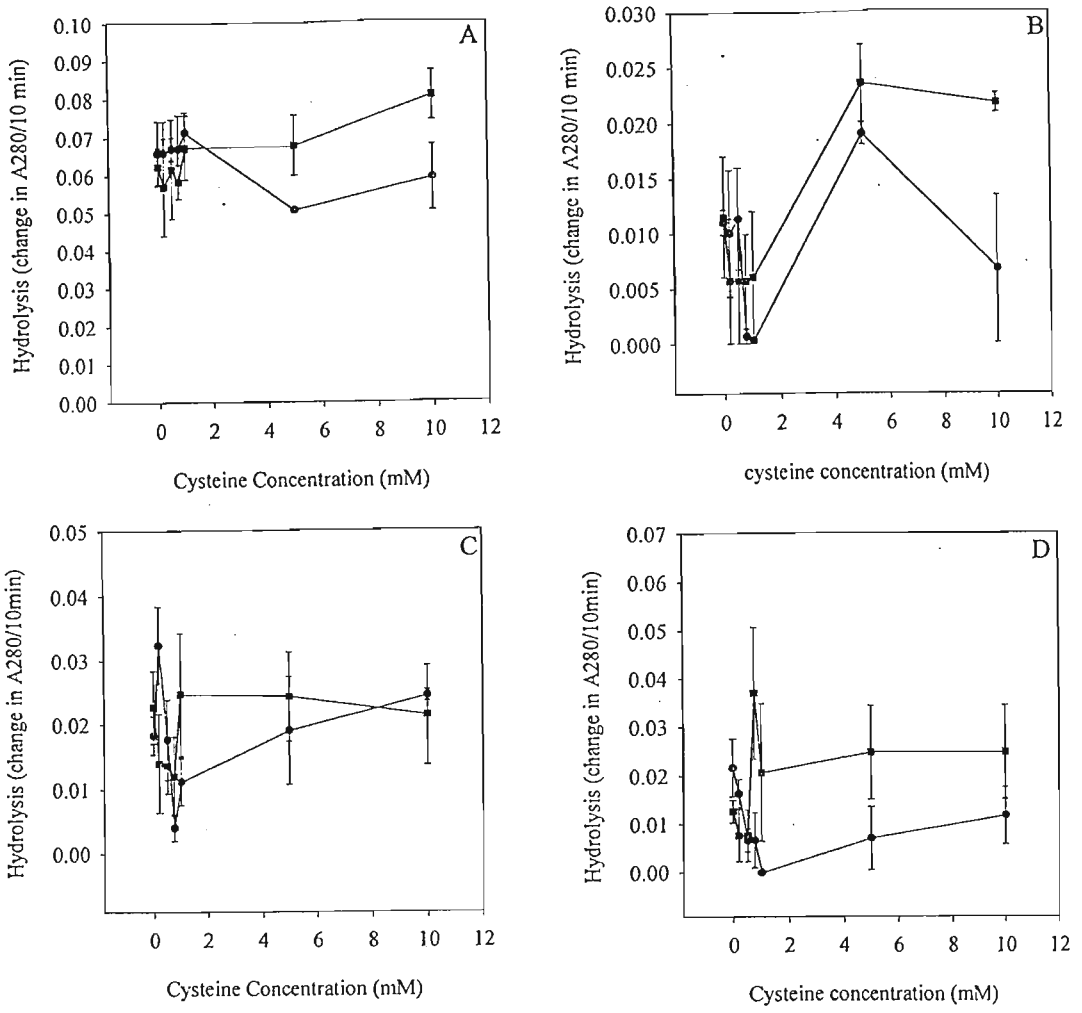

Figure 5.8. The effect of cysteine:cystine AMT redox buffers on cathepsin D hydrolysis of haemoglobin (A), RA-BSA (B), BSA (C) and lysozyme (D) was assessed at pH 6.0. The redox buffers contained cysteine (0-10 mM) and cystine at 0 mM (●) and 0.8 mM (■). The data represents the mean ± standard error of 3 independent experiments.

5.5 Discussion

From previous work in this laboratory and others, the activity and stability properties of cathepsin B appeared to be disparate. Activity appeared to have a neutral to slightly basic pH optimum, and yet the enzyme was most stable at a more acidic pH. In the present study, however, the activity of cathepsin B was similar at pH 6.0 and pH 7.0. This may be due to differences in the assay and cathepsin B isolation procedures used in these experiments. Isolated cathepsin B is usually not made up of fully active enzyme. Further the enzyme may be oxidised, and has to be reduced to restore activity. In the present study cathepsin B was affinity purified, and the enzyme was pre-activated before being used in the respective (pH) test buffers. In previous experiments, less than 100% active enzyme may have been used and/or the enzyme was activated in the test buffers themselves. In

these cases, differences in activity at different pHs may reflect the degree of reduction at the different pHs. Since reduction occurs more efficiently with increasing pH, the degree of reductive activation, and hence activity, would be expected to be greater at pH 7.0 than at pH 6.0 for a given concentration of thiol. For this reason, perhaps, cathepsin B appeared to have a neutral pH optimum.

In contrast to the activity of the enzyme in the present study, the *stability* of cathepsin B was found to be significantly greater at pH 6.0 than at pH 7.0. This suggests that the enzyme would be capable of operating at maximal activity at pH 6.0, without the loss of stability that occurs at higher pH. Thus, it is considered likely that *in vivo,* cathepsin B generally operates at acidic, and not neutral pHs. The enzyme showed local minima in activity in cysteine (pH 5.0-7.0) and GSH-containing buffers, as well as in stability, in cysteine-containing buffers (pH 6.0 and 7.0). A common mechanism may be responsible for these minima. Krepela *et al.* (1999) suggested that the local minimum in cathepsin B activity with increases in cysteine concentration is due to cysteine-thiol inhibition of the enzyme. However, the results described here suggest that this does not adequately explain this phenomenon. A further potential flaw in the study of Krepela *et al.* (1999) work is that the ratio of thiol:thiolate remains essentially the same with increases in cysteine concentration, at a given pH. Thus, the thiolate concentration does not increase relative to the thiol concentration, and therefore the levels of cathepsin B inhibition should have been the same for any concentration of cysteine at a given pH. The local minimum in activity is not present in buffers containing cystine.

GSH is the principal cytosolic reducing agent in eukaryotes, with concentrations ranging from 0.1-10 mM (Allen, 1991; Meister, 1995). It appears that the activity of cathepsin B is depressed in GSH-containing buffers, compared to cysteine-containing buffers at pH 7.0. The high cytosolic GSH concentration may thus restrain cathepsin B activity in the cytoplasm. This may provide an additional defence, should cathepsins leak from 'lysosomes' (Brunk *et al.*, 1995).

The effect of cysteine:cystine redox buffers on cathepsin B activity was assessed at pH 6.0 and pH 7.0. In these experiments, it was found that the enzyme activity did not change over a broad redox potential range of -80 mV to -350 mV, i.e. from relatively oxidising conditions to relatively highly reducing conditions. The endoplasmic reticulum, for example, is considered to be a relatively oxidising environment, and has an estimated redox

potential between -170 and -185 mV (Hwang *et al.*, 1992). In our experiments, cathepsin B was also assayed under relatively highly reducing conditions (< -250 mV), and the enzyme activity was not affected. Thus, our data indicates that cathepsin B activity is similar in both relatively oxidising and reducing environments. These results suggest that the activity of cathepsin B is probably not redox regulated *in vivo*.

The stability of cathepsin B was also measured under varying redox conditions. At pH 6.0, the enzyme was essentially stable to all redox conditions tested within the limits of these assays. At pH 7.0, however, it was found that the stability was linearly related to redox potential up until -250 mV (Fig. 5.5). The absolute concentration of cystine affected stability, with higher concentrations of cystine depressing stability at a given redox potential. The negative effect of cystine and oxidising redox conditions on the stability of the enzyme at pH 7.0, could occur through two possible mechanisms. Firstly, cystine and oxidising redox conditions may accelerate the pH-dependent denaturation of the enzyme. Alternatively, cystine-dependent oxidative denaturation of the enzyme may occur more efficiently at pH 7.0 than at pH 6.0, as thiol-disulfide exchange occurs more efficiently at higher pH. These two mechanisms are not mutually exclusive. Thus, despite cathepsin B activity showing no sensitivity to redox potential at pH 7.0, the stability of the enzyme may be affected by redox potential and the ambient concentration of cystine.

These experiments indicate that cathepsin B activity may not be redox regulated as the enzyme is capable of operating over a broad redox potential. As the endolysosomal redox environment may be highly dynamic, it appears that cathepsin B may be well-suited to the system. Could cathepsin B operate in extra-endolysosomal redox environments, and would it be stable? The present study indicates that the activity of the enzyme does not preclude it from operating in redox environments other than the endolysosomal system. However, the pH, and the nature and ratios of redox agents present within these environments, may affect the stability of the enzyme.

Most whole protein substrate hydrolysis assays have, in the past, been undertaken without protection against oxidation. Hence, the effect of oxidised forms of reducing agents on substrate hydrolysis has not been reported. In the present study, cathepsin B hydrolysis was undertaken with cystine at differing levels. All assays were conducted at pH 6.0, as the fluorometric assay data indicated that the operational pH for cathepsin B *in vivo* may be acidic.

The results indicate that the degree of hydrolysis of whole protein substrates in AMT redox buffers depends on whether the substrate has disulfide links or not. In those substrates without disulfide bridges (haemoglobin and RA-BSA), cystine appears to increase the rates of hydrolysis in a dose-dependent manner. This effect is not due to cathepsin B (Fig. 5.4), nor is it due to the effect of cystine on the substrates alone (control experiment, Section 5.3.3). It appears therefore that cystine may exert this effect on the protease-substrate interaction. However, in those substrates with disulfide links (BSA and lysozyme) cystine does not appear to exert this effect on proteolysis, although, lysozyme hydrolysis may be stimulated by 0.2 mM cystine. It should be noted that lysozyme has four disulfide links compared to the seventeen disulfide links of BSA (Table 5.1). To confirm this effect, the effect of cystine on the hydrolysis of these substrates by cathepsin D was undertaken. Cathepsin D is an aspartic protease, and operates at relatively acidic pHs compared to cathepsin B. However, even at these low pHs cystine appeared to stimulate hydrolysis of substrates without disulfide bridges. Thus, this data confirms that this effect is not due to cathepsin B alone, and may be a general feature of proteolysis in these buffers. In the case of all substrates tested, there was no correlation between substrate hydrolysis and redox potential.

Two questions arise: what is the mechanism behind this effect, and, is it relevant *in vivo*? The mechanism by which cystine exerts its effects, may not be redox related. In those substrates without disulfide links, or even available cysteine residues, cystine increased the rates of proteolysis. Cystine may exert this effect by affecting the structure of the substrates during degradation; or by influencing the enzyme-substrate interaction. It has been shown that cystine alone is capable of refolding oxidised-denatured lysozyme (Raman *et al.*, 1996). These authors also found that the cysteine:cystine redox pair was more effective than a GSH/GSSG redox buffer system in refolding lysozyme. It may be possible that a cysteine: cystine redox system may influence *available* substrate concentration, thereby increasing the rates of proteolysis. In the cases of those substrates with disulfide links, cystine may also exert a similar effect. However, in addition, cystine may decrease proteolysis by hindering disulfide bond breakage. This could explain the partial increase in proteolysis, in buffers containing cystine, of lysozyme (4 disulfide bridges), compared to BSA (17 disulfide bridges). A feature of the effect of cystine is that it also stimulated cathepsin D hydrolysis of substrates that had no disulfide bridges. These assays were done

at a very acidic pH (pH 3.2), where redox reactions would be expected to occur very inefficiently, supporting the contention that cystine's effect may not be redox related.

Is this relevant *in vivo*? Unfortunately, the levels and ratios of cysteine:cystine within the endolysosomal system have not been determined. However, it is clear from the results reported here that hydrolysis of these protein substrates does not depend on redox potential. Collectively the data indicate that redox potential *per se* does not appear to have a significant effect on proteolysis *in vivo*. The endolysosomal redox environmnet may, however, exert an effect on cathepsin B stability and on protease-substrate interactions.

CHAPTER 6

THEORETICAL CONSIDERATIONS REGARDING POSSIBLE THERAPY OF PATHOLOGIES BASED ON LYSOSOMAL PROTEASES

6.1 Introduction

The bulk of this thesis has been concerned with the action of proteases within the endolysosomal system. A systemic approach was adopted in looking at protease regulation within the endolysosomal system. In certain pathological conditions like cancer, these proteases may be secreted and contribute to the destruction of extracellular components (Yan *et al*, 1998). Looking ahead to the possibility of controlling conditions that involve inappropriate protease activity, some concepts on the use of protease inhibitors as therapeutic agents were reviewed and are discussed in this chapter. As with the endolysosomal system a system-based approach, rather than a moleculer-based approach was adopted.

There appear to be at least two major considerations for using a protease inhibitor as a therapeutic agent: the inhibitor should be effective, and should have good pharmokinetic properties. In order to fulfil the latter consideration the inhibitor should have minimal peptide character, high stability, good membrane permeability, good retention within the body, good bioavailability and should usually be less than 1 kDa in size (Leung *et al.*, 2000).

In order for the inhibitor to be effective *in vivo* it should inhibit the target protease rapidly, and this interaction should be stable. In most cases, specific inhibitors are favoured. However, in some cases, class specific inhibitors are favoured. The inhibitor should also be reversible, as irreversible inhibitors tend to have highly reactive moieties that may interact non-specifically with other electrophiles or nucleophiles found within the body (Leung *et al.*, 2000). This discussion will focus on the choice of inhibitors for therapeutic purposes.

Traditionally, natural products were screened for their inhibitory potential and empirical methods were used to design an inhibitor with the appropriate character. However, this screening procedure is being replaced by a new approach known as 'rational drug design'. Using this approach, at least three strategies are used to find potential therapeutic drugs

(Leung *et al.*, 2000). Firstly, the natural or perhaps the 'best' substrate of the protease is used as a framework to design a peptide inhibitor. This inhibitor usually has a transition state isostere to trap the enzyme catalytic mechanism in some transition state (Leung *et al.*, 2000). The peptidic character of the inhibitor is then reduced to ensure that the inhibitor has good pharmokinetic properties. A second approach is to use the crystal structure of the protease or a closely related enzyme to design an inhibitor that fits directly into the active site of the enzyme (Leung *et al.*, 2000). Saquinavir, an inhibitor of the HIV-1 protease, was the first drug, designed in this way, to be approved for human use (Pakyz and Israel, 1997; Patick and Potts, 1998). Finally, combinatorial chemistry is used to generate a library of chemical compounds that are screened for their inhibitory nature (Leung *et al.*, 2000). In most cases the screening protocols used, select for competitive-type inhibitors.

Using these approaches, there have been some successes, notably for protease inhibitors directed at the HIV-1 protease, especially when these inhibitors are used in combination with other drugs (Leung *et al.*, 2000). There have also been some failures, notably for metalloprotease inhibitors in the treatment of cancers. The reasons for the failure of these drugs could be attributed to poor clinical trial design, an incomplete understanding of the *in vivo* function of these proteases, and the fact that these inhibitors are designed only to be cytostatic (Coussens *et al.*, 2002).

6.2 *In vitro* parameters used for selecting protease inhibitors

How are good protease inhibitors selected? There are two considerations for selecting a protease with potentially good inhibition properties *in vivo*. Firstly, the inhibitor's concentration *in vivo* should be known. Secondly, the inhibitor should have good inhibitory characteristics, which are usually measured *in vitro*. Bieth (1984, 1995) related these *in vitro* characteristics with the inhibitor's potency *in vivo*. The inhibitor should rapidly inhibit the target protease, and secondly the enzyme inhibitor complex should be stable. Assuming that the *in vivo* inhibitor concentration is significantly greater than the protease concentration, and that the inhibitor behaves in an irreversible or pseudoirreversible manner, the time that it takes a protease-inhibitor association to be >99% complete is given by the delay time of the reaction:

$$d(t) \cong \frac{5(1+\frac{(S_o)}{K_m})}{k_{ass}(I_o)} \quad (3)$$

where $d(t)$ is the delay time of inhibition, (S_o) is substrate concentration, K_m is the Michaelis constant for that substrate, k_{ass} is the rate constant for enzyme inhibitor reaction, and (I_o) is the inhibitor concentration (Bieth, 1984). If there are many substrates that compete with the inhibitor for the enzyme, their affects are additive, i.e.

$$d(t) \cong \frac{5\sum_1^N (1+\frac{(S_{oN})}{K_{mN}})}{k_{ass}(I_o)} \quad (4)$$

The k_{ass} may be measured using *in vivo* substrates and then the delay time for the reaction is given by:

$$d(t) \cong \frac{5}{k'_{ass}(I_o)} \quad (5)$$

where k'_{ass} is the *in vivo* association rate constant (Bieth, 1984). The delay time of inhibition can then be used to determine the effectiveness of an inhibitor *in vivo* using an equation that measures the degree of substrate turnover in the presence of an inhibitor:

$$\frac{(P_\infty)}{(S_o)} = \frac{k_{cat}}{K_m}.(E_o)\frac{d(t)}{5} \quad (6)$$

where (P_∞) is the product concentration at the end of the inhibition process, k_{cat} is the catalytic rate constant, E_o is the enzyme concentration (Bieth, 1984). Using this measure, it can be shown that with a $d(t)$= 1 sec, no more than 2% of substrate will be consumed before inhibition.

Bieth (1984) also determined that the stability of the proteinase-inhibitor complex depends on the ratio of $(I_o)/K_i$. If this ratio is greater than 1000, then a reversible inhibitor may display pseudoirreversible inhibition, and is stable to substrate-induced dissociation of the protease-inhibitor complex. Further, the effect of competing substrate may be minimal. The design of proteinase inhibitors is therefore centred on minimising the delay time of inhibition, and increasing the ratio of $(I_o)/K_i$.

In summary, to assess a protease for good *in vivo* inhibition properties, the following parameters have to be checked. The K_i or apparent K_i (K_{iapp}) of the protease-inhibitor reaction must be determined, and the *in vivo* concentration of the inhibitor should be known. Secondly, the delay time of inhibition should be determined. If this is less than 1 sec, it is reasonable to assume that the protease-inhibitor interaction occurs rapidly enough to prevent much substrate degradation. If the $(I_o)/K_i$ ratio is >1000, it is likely that the inhibitor behaves in a pseudoirreversible manner towards the protease, and the effect of competing substrate is negligible. Finally, although not discussed above, the stability of the protease-inhibitor interaction must be assessed. This general approach is employed in screening for protease inhibitors as potential therapeutic agents.

6.3 Protease-inhibitors interactions: the effect of E_o

Enzyme inhibitor complex formation also shows some particular properties. It can be shown that protease-inhibitor interactions can display either tight-binding or irreversible or classical behaviour depending on the ratio of E_o/K_i (Fig. 6.1) (Bieth, 1984). If the ratio of E_o/K_i is very high, then the inhibitor might actually titrate the protease (curve 1, Fig. 6.1) Here, the inhibitor concentration is approximately equal to the protease concentration and can form equimolar complexes.

If, however, the ratio of E_o/K_i is very low (for the same protease:inhibitor interaction), then the behaviour of the protease and inhibitor is called 'classical'. Under classical conditions, [inhibitor] >> [protease], and the resulting curve has an exponential shape (curve 4, Fig. 6.1). The effect of K_i, [protease] and [inhibitor] has two important implications. Firstly, in terms of rational drug design, inhibitors should be designed with as low a K_i as possible to ensure that they may operate in titration rather than classical conditions. The second implication is that for a given inhibitor (with a fixed K_i), the nature of the interaction between the protease and inhibitor may depend on the concentration of the protease. For example, if the enzyme concentration relative to the K_i is very high (curve 1, Fig. 6.1), then titration-type behaviour will be expected. If, however, the enzyme concentration is low relative to the K_i (curve 6, Fig. 6.1), then inhibition may be described as classical or even not be detected.

If these properties hold *in vivo*, it does suggest a rather elegant mechanism in which limited proteolysis is allowed, but large concentrations of protease are efficiently inhibited.

Assuming that the [inhibitor] >> [E_o], as the protease concentration increases, the nature of the protease-inihibitor interaction changes from 'classical' to 'titration' (from curve 6 to curve 1, Fig. 6.1).

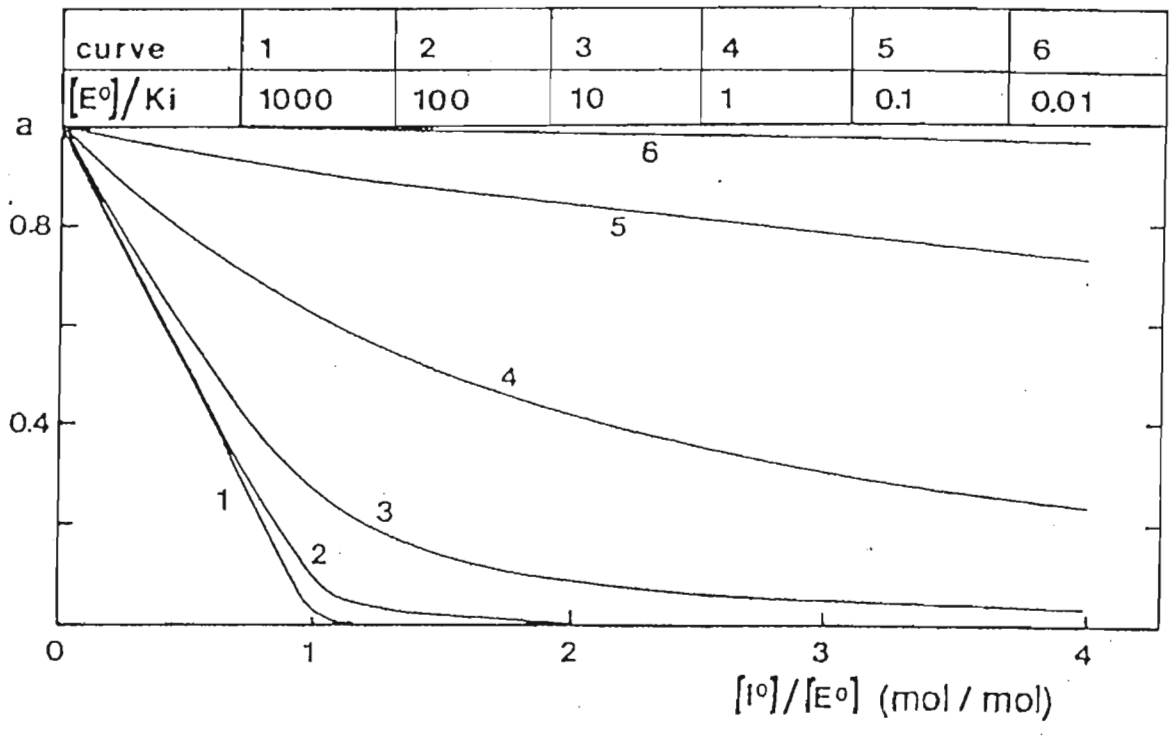

Figure 6.1. The effect of the variable [E_o]/K_i on fractional free enzyme. Plots were constructed using the Morrison equation (from Bieth, 1995).

This also suggests a mechanism through which disregulated proteolysis could occur. If relatively high concentrations of protease were secreted by tumour cells or pathogenic organisms then titration conditions ([E_o]/K_i > 100) would prevail, effectively stopping inappropriate proteolysis. If, on the other hand, tumour cells, or other pathogenic organisms, continually secrete small amounts of protease such that the ratio [E_o]/K_i remains less than 10, classical conditions between the protease and inhibitor may prevail (Figure 6.1), and thus some of these proteases may escape inhibition. Whilst it may be argued that small amounts of protease may not significantly damage host tissue, the continuous secretion of a protease may yet result in significant damage to host tissue, whilst circumventing host protease inhibitors. As an inhibitor may operate under classical or titration conditions depending on the ratio of protease to inhibitor, therapeutic inhibitor intervention in conditions where disregulated proteolysis occurs may require the

concentration of the target protease to be known. Alternatively the maximal permissable [protease] may need to be known.

6.4 Metabolic control analysis and inhibition *in vivo*

The class of protease inhibitors favoured for therapeutic applications are usually of the competitive type. However, Cornish-Bowden (1995; 1999) has described how effective uncompetitve inhibitors may be *in vivo*, especially compared to competitive-type inhibitors. Enzyme-substrate-inhibitor interaction *in vivo* may be different to the situation *in vitro* (Cornish-Bowden, 1995;1999). Usually the kinetic properties of an enzyme are studied by measuring the rate of the reaction using various substrate concentrations, i.e. fixed substrate concentrations and variable rates. This data is then modelled on the Michaelis-Menton equation, usually using non-linear regression analysis. The situation *in vivo* may be essentially the opposite. Enzyme systems may adjust appropriate substrate concentrations to achieve a particular rate, i.e. fixed rates and variable substrate concentrations (Cornish-Bowden, 1995; 1999). This situation might prevail *in vivo* for most enzymes (Cornish-Bowden, 1995). Thus the Michaelis-Menton equation can be derived in terms of substrate rather than initial rate (Cornish-Bowden, 1995):

$$S_o = \frac{K_m v_o}{V - v_o} \tag{7}$$

Using this equation, expressions for uncompetitive competition and competitive competition are given below (Cornish-Bowden, 1995; 1999). For competitive inhibition, the rate equation (Cornish-Bowden, 1995) is given by:

$$S_o = \frac{K_m(1 + \frac{i}{K_i})}{\frac{V}{v_o} - 1} \tag{8}$$

For uncompetitive inhibition, the expression (Cornish-Bowden, 1995) is given by:

$$S_o = \frac{K_m}{\frac{V}{v_o} - 1 - \frac{i}{K_{iu}}} \tag{9}$$

Comparing these two expressions, it can be demonstrated that there is no finite value for i where competitive expression could yield zero substrate concentration, as both i and S_o

share a linear relationship. Further, modelling of the equation by Cornish-Bowden (1995; 1999) revealed that the effects of competitive inhibition may be overcome by relatively moderate changes in the substrate concentration. On the other hand, it is clear that the denominator of the expression for uncompetitive inhibition can be zero, suggesting that steady-state (between the enzyme and substrate) may never be reached (Cornish-Bowden, 1995). Thus, uncompetitive inhibitors are expected to be extremely potent *in vivo*.

The effectiveness of these inhibitors *in vivo* may be due to the fact that their action is actually potentiated by substrate, making them highly efficient inhibitors. In contrast, competitive inhibitors compete against available substrate and therefore have to be present at significantly higher concentrations compared to uncompetitive inhibitors. This also has implications on the cost and pharmokinetic properties of competitive versus uncompetitive inhibitors. Uncompetitive inhibitors may be more cost-effective as they would be expected to be effective at lower concentrations than competitive inhibitors. Further, as they may operate at lower *in vivo* concentrations than competitive inhibitors, the optimisation of their pharmokinetic profiles may be simpler.

The efficiency of uncompetitive inhibition has been demonstrated for the herbicide 'Round-up' (glycophosphate) which inhibits 3-phosphoshikimate 3-carboxyvinyltransferase, as well as for Li^+ which inhibits *myo*-inositol monophosphate (used to treat manic depression) (reviewed by Cornish-Bowden, 1999). Brefeldin A has recently been shown to be an uncompetitive inhibitor of ADP ribosylation factor-1 (or Arf-1) exchange factors which are important for secretion by mammalian cells (reviewed in Chardin and McCormick, 1999). It is clear that not all the enzymes inhibited uncompetitively are metabolic-type enzymes, and thus the effectiveness of uncompetitive inhibitors is not limited to these enzymes. Uncompetitive protease inhibitors have not been extensively reported in the literature, but non-competitive inhibition of the trypanosomal protease oligopeptidase-Tb by suramin was demonstrated (Morty *et al.*, 1998). Non-competitive inhibitors have an uncompetitive component to them, and the effectiveness of suramin was demonstrated by its high trypanocidal activity *in vivo* (Morty *et al.*, 1998). Uncompetitive inhibitors may be rarely found *in vivo*, in part due to their high efficiency (Cornish-Bowden, 1995). However, as long as an inhibitor has a significant uncompetitive component, it may be highly effective *in vivo* (Cornish-Bowden, 1995; 1999). It may be worthwhile for inhibitor screens to be modified to look beyond *in vitro* parameters such as K_i, $(I_o)/K_i$ and the delay time of

inhibition. The screens could also take into account the type of inhibition, or the contribution made by uncompetitive-type inhibition. Uncompetitive protease inhibitors may provide a new, highly efficient class of inhibitors for therapeutic intervention in diseases involving proteases. Instead of looking at the contours of the active site, researchers could look at the contours of the ES complex and try to rationally design compounds which would complex this. One possibility would be to use a substrate analogue which forms an irreversible ES complex and use this to model the contours. Alternatively uncompetitively inhibiting antibodies may be raised to the ES complex.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

In the past, proteolysis within the endolysosomal system was studied by two different schools. The proteases within the endolysosomal system were isolated, and their kinetic and structural properties determined *in vitro*. Alternatively, proteolysis within the endolysosomal system was approached using traditional cell biology methodologies like electron microscopy and organelle fractionation by density gradient centrifugation. Those studies that managed to use tools from both approaches often yielded the most interesting results (e.g. Tjelle *et al.*, 1996). In this thesis, an effort has been made to integrate knowledge of the endolysosomal system (cell biology), with the properties of the enzymes (enzyme kinetics). The approach was found to be useful, as it gave insights into how the entire endolysosomal system may be regulated. An example of this is the proposed mechanism for protease aggregation within lysosomes (Section 2.1). Hydrolases within lysosomes may aggregate due to electrostatic interactions with LAMP-2 at low pH. In trying to isolate lysosomal enzymes, their strong cationic properties are often used to separate them by ion-exchange chromatography (Chapter 4). Thus, it was possible to associate the proteases' electrostatic properties with regulation *in vivo*. Also, this mechanism may explain the functional differences between late endosomes and lysosomes, despite their similar constituents. The electrostatic properties of these proteases may also play a role in the organelle distribution of these enzymes. As can be seen in Table 2.2, the pI of cathepsin H > cathepsin S > cathepsin B and L. Cathepsin H may be predominately localised to the early endosome, whilst cathepsin S is predominately located in the late endosome. Cathepsins B and L have a late endosome-lysosome distribution. It is thus possible that retention of a protease within a specific organelle along the endolysosomal system is dependent on the pI of that protease.

7.2 Review of the endolysosomal system

In an effort to integrate knowledge of the endolysosomal system with our current understanding of lysosomal proteases, it was necessary to develop a comprehensive view of

the system, as this helped focus the research efforts, and facilitated interpretation of the results in the context of the endolysosomal system as a whole. To this end the endolysosomal system was reviewed, concentrating on proteolytic environments with the system. Some important results were obtained from this review. Firstly, it became clear that the proteases within the endolysosomal system are regulated in a manner that does not involve inhibitors. Rather, the proteases may be subjected to 'organelle-dependent' regulation of proteolysis, in a hierarchical system.

The principal proteolytic environments within the endolysosomal system are the late endosome (or the hybrid organelle), the phagosome and autophagosome. Lysosomes are believed to be storage organelles for the proteases, and the bulk of proteolysis does not occur within these organelles. The proteolytically competent organelles, have a common set of molecules that appear to be necessary for proteolysis: the proteases themselves, the v-ATPase, LAMPs and perhaps the cysteine transporter (Chapter 2). The v-ATPase and cysteine transporter may effect changes in the luminal conditions within these organelles, affecting both the substrates and proteases within the system. Disulfide links within the structure of substrate may be reduced either by the cysteine itself, or the cysteine-dependent thiol reductase GILT. Acidification may also aid proteolysis by also affecting substrate structure. The pH and redox conditions would also affect the activity and stability of the 'lysosomal' hydrolases within the endolysosomal system but these appear to be well adapted to these conditions, having half-lives from days to weeks.

As demonstrated in Table 2.1 (Chapter 2), not much is known about the importance of the lysosomal cysteine transporter in autophagy or phagocytosis. The sequence and structure of the transporter is also not known. In fact, not much is known about the sequences of most of the 'lysosomal' amino acid membrane transporters. To date, only a single transporter for small and neutral amino acids has been sequenced (Sagné *et al.*, 2001). Using human genome data (www.ncbi.nih.gov), and bioinformatics tools (like the Basic Local Alignment Search Tool or BLAST), a search of the database was undertaken for the cysteine transporter. Several search criteria were used, including known lysosomal targeting signals like GYXXΦ, where Φ is a hydrophobic amino acid residue (Gough *et al.*, 1999), or the dileucine motif (Johnson and Kornfeld, 1992). Another approach used, was to identify cysteine transporters found in other organisms. These were then used to do BLAST searches through the human genome database. These potential cysteine transporters were

screened using structure prediction programmes from Swiss-Prot (www.expasy.ch) and manually looking for lysosomal targeting signals. Potential putative cysteine transporters were used to do BLAST searches through other databases to check whether they showed homology to other cysteine transporters. Unfortunately the human genome database currently has too many 'unknown' proteins for a function to be assigned, based on their sequences. Whilst the search itself was unsuccessful, it raised some interesting points about the cysteine transporter. Cysteine is not favoured as a reducing agent in organisms that exist under aerobic conditions, as it is oxidised rapidly by atmospheric oxygen (Newton and Fahey, 1995). Cysteine is, however, found as a major biothiol in organisms growing under anaerobic conditions. For organisms growing under aerobic conditions, GSH, which oxidises more slowly, is preferred (Newton and Fahey, 1995). As a result, cysteine transporters, and lysosomal cysteine transporters in particular, may not be as common in nature as other amino acid or biothiol transporters. Once the transporter has been isolated and sequenced, the role that it has played in the evolution of endolysosomal function may be a fascinating research topic.

The transporter is believed to support reducing conditions within the latter stages of the endolysosomal system. The redox potential within the system, and its effect on proteases and their substrates has not been determined. In this study an attempt was made to determine the redox properties of a lysosomal cysteine protease, cathepsin B, and to extrapolate these results to the possible effect of reducing conditions *in vivo*.

7.3 Redox properties of cathepsin B

Before the properties of cathepsin B could be characterised, an efficient isolation procedure was needed. In the past our laboratory has utilised TPP as an upstream step in the isolation of proteases. In this study it was discovered that, by including 30% *t*-butanol in homogenisation buffers, lysosomal hydrolases were placed in 'suspended animation', preventing artefact formation and improving yields (Dennison *et al.*, 2000). Affinity columns were used to ensure that the downstream purification procedures were efficient and yielded a high proportion of active enzyme (80-100% for cathepsin B). It was expected that the proportion of active enzyme in redox assays would be important, as redox buffers have the capacity to refold/reactivate oxidatively denatured enzyme (Raman *et al.*, 1996).

The assay procedures used in the redox experiments were also novel. Cathepsin B was isolated in an inactive complex, but was pre-activated before use. This generally yielded close to 100% active enzyme molecules, by E-64 titration. In cathepsin B preparations that contain only 30-50% active enzyme, inactive molecules may be renatured by the redox buffers. It has been shown that redox potential and pH share an inverse relationship (Segal, 1976). Thus at higher pH the greater efficiency of reduction may increase the proportion of active enzyme molecules. This will give higher apparent activities at higher pH and an erroneously high apparent pH optimum. This cannot happen if 100% active enzyme is used. Further, although pre-activating cathepsin B is a standard assay procedure, most pH-optimum studies pre-activate oxidised cathepsin B in the test pH buffer. Thus, when determining the pH optimum of cathepsin B, the efficiency of reduction may be greater at a higher pH than a lower pH for a given concentration of thiol. This would also tend to give an apparently higher pH optimum for cathepsin B. In this study, cathepsin B was always pre-activated in the same assay buffer to ensure that the enzyme used during the assay procedures was always between 80-100% active. Finally, and in contrast to most other cathepsin B assay procedures, care was taken to limit oxidation of the buffers by atmospheric oxygen.

Some significant results were obtained from these experiments. In the past cathepsin B was assigned a neutral pH optimum for activity (Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992; Dehrmann *et al.*, 1996). However the enzymes' stability was apparently greater at acidic rather than neutral pH values (Dehrmann *et al.*, 1996). The results obtained in the present study suggested that this paradox may be an artefact created by not using 100% active enzyme. The present data indicates that both the enzymes' stability and activity were optimum at acidic pH. Thus, the operational pH of the enzyme *in vivo* may be acidic. It was also found that the enzyme showed a local minimum in activity with increasing concentrations of cysteine. This decrease in activity was abrogated by increasing cysteine concentration. This effect was found over all pHs tested, and with GSH-containing buffers. Further, the enzymes' stability showed a similar response to increasing cysteine concentration. This data indicates that the proposed inhibition of cathepsin B activity by the thiol form of cysteine (Krepela *et al.*, 1999) is unlikely. The enzyme may become less stable, although the mechanism, and significance of this effect *in vivo*, is unclear. This local minimum effect on cathepsin B activity was not present in buffers that contained cystine.

With buffers containing cysteine and cystine, it was possible to calculate the redox potential and the effect of cystine on cathepsin B activity and stability was assessed. This is the first time, to the author's knowledge, that the redox properties of any cysteine protease has been probed. It is often assumed that cysteine proteases operate under reducing conditions, but these conditions are usually not defined. The results obtained using a synthetic substrate indicated that cathepsin B stability, but not activity was affected by redox potential at pH 7.0. At pH 6.0, cathepsin B was too stable for any effect of redox potential to be determined. However, the activity of the enzyme did not show any dependence on redox potential over all pHs tested. Cathepsin B activity, therefore, is probably not redox regulated *in vivo*. However, reducing conditions may stabilise the enzyme at higher pH as the late endosome/lysosome pH environment may vary to support hydrolases with basic pH optima (Tjelle *et al.*, 1995).

The effect of cysteine/cystine redox buffers on cathepsin B activity against whole protein substrates was also determined. It was found that the hydrolysis of substrates without disulfide bridges appeared to be stimulated by cystine. In contrast, for substrates with disulfide bridges, cystine gave mixed results. Similar results were obtained with cathepsin D, an aspartic protease that operates at very low pHs. Control experiments indicated that the effect of cystine on cathepsin B hydrolysis of these substrates was not due to the enzyme alone, or to degradation of the substrate by the buffer. Rather, it may involve the interaction of enzyme and substrate. This may not necessarily be redox dependent, as the effect of cystine was greatest on those substrates without disulfide bridges. BSA, which has seventeen disulfide bridges showed no stimulation, whilst lysosome, which has two disulfide bridges, showed partial stimulation of hydrolysis, Haemoglobin and reduced-alkylated BSA, which lack free cysteine residues, showed definite increases in hydrolysis in the presence of cystine. The stimulation of cathepsin D hydrolysis of whole protein substrates, showed that these effects were not limited to cysteine proteases. As redox reactions would be expected to occur inefficiently at low pH, the results obtained using cathepsin D support the view that the effect of cystine is not redox dependent (Chapter 5). For none of the substrates tested, under the conditions employed in these experiments, did the rates of substrate hydrolysis show any relationship with redox potential. Perhaps future research could be directed at exploring the effects of cysteine:cystine redox buffers on structure and folding for proteins lacking free thiols

groups or disulfide bridges. Once these effects are quantified, perhaps the results presented here can be explained.

The relevance of these results *in vivo* is difficult to assess as components of the 'lysosomal buffer' have not been discovered yet, nor has the intralysosomal redox potential(s) been determined. Proteolysis within the endolysosomal system may be more complicated than is often assumed. 'Lysosomes' have been shown to have heat-shock proteins (Cuervo and Dice, 1998), and GILT (Arunachalam *et al.*, 2000). In many ways, destruction of whole protein substrates within the endolysosomal system may be the reverse of protein folding within the ER. The redox buffer, and proteins that affect protein structure directly may therefore be important within the endolysosomal system. Future research efforts could be directed at isolating the lysosomal cysteine and cystine transporters, determining the endolysosomal redox potential(s), and the effects, if any, of these on protein hydrolysis *in vivo*.

7.4 Hierarchical organisation of the endolysosomal system

There are several possible regulation points for hydrolysis within the endolysosomal system (Figure 2.1). Of these, the signalling networks may play the largest role in controlling the rate-limiting steps in endolysosomal hydrolysis and may exert their influence through molecules that interface between the signalling and endocytic pathways. Some progress has been made in uniting the signalling networks, the regulation of endolysosomal fusion reactions, and the components that affect the luminal endolysosomal proteolytic environment into an integrated model for proteolysis. Recently it has been shown that p38 MAP kinase may accelerate the rate of endocytosis in response to oxidative stress (Cavalli *et al.*, 2001), providing a direct link between the endocytic rate and the signalling pathways. It has also been shown that the E subunit of the V-ATPase may interact with the DH domain of mSos1, a guanine nucleotide exchange factor involved in Rac 1 activation (Miura *et al.*, 2001). Rac 1 activation regulates various trafficking events like transferrin receptor mediated clathrin coat vesicle formation (Lamaze *et al.*, 1996). Although in this case, the E subunit affected the mSos1-Rac1-signalling pathway (Miura *et al.*, 2001), it does indicate that there are domains on the V-ATPase that are capable of interacting with signalling pathways. The activity of chloride ion channels that influence luminal pH, is regulated by protein kinase A-dependent phosphorylation (Forgac, 1998). Thus, it is possible that

acidification of endolysosomal organelles may be connected to these signalling pathways. Thus, regulation of conditions within endolysosomal organelles, and therefore proteases, may be integrated via signalling networks, to endocytosis and changes occurring at the cellular level.

Endolysosomal proteolysis has previously been viewed in the following manner: substrate is channelled into 'lysosomes', where active lysosomal hydrolases are present at high concentrations. At these high concentrations, substrate is essentially limiting, and would be hydrolysed very rapidly. No distinction was made between the proteolytic environments within the endolysosomal system. In this bulk, non-specific hydrolysis model, no consideration has been given to regulation of proteolysis, feed-back inhibition, or fluxes through the endolysosomal system. Essentially lysosomal proteolysis has been viewed as 'garbage disposal', with no luminal regulatory steps necessary.

In the review given in Section 2.1, it was recognised that the endolysosomal system is made up of functionally distinct, but related environments. In terms of proteolysis, the most important of these relationships is between lysosomes and late endocytic components, i.e. maturing phagosomes, AVds, and late endosomes. Lysosomes, despite containing the highest concentrations of proteases within the endolysosomal system, are not the main site of proteolysis. Proteolysis occurs primarily in pre-lysosomal compartments, suggesting that there may be regulation of proteolysis, i.e. the luminal conditions within the different organelles may support proteolysis or not.

Creation of a proteolytically competent environment within the endolysosomal system depends of the acquisition on certain components: a v-ATPase, proteases, LAMPs, and perhaps the cysteine transporter. These components may be acquired by fusion between organelles and the creation of proteolytically competent environments may thus also be controlled by regulating the fusion between organelles within the system. For instance, fusion with lysosomes may be crucial to introducing the necessary proteases to create proteolytically competent environments. Also, as substrate may be limiting within the endolysosomal system, the channelling of substrate from the early endosome to the later endocytic organelles may be important. What controls the rate at which organelles within the endolysosomal fuse and/or reform? Is the entire endolysosomal system regulated, and if so how? Unfortunately most studies of proteolysis within the system have been largely descriptive in nature, detailing where proteolysis occurs but not dealing with the kinetics of

the system. Also, most studies have not considered whether the proteases within the system manifest systemic properties.

If we consider the endolysosomal system from proteases through to the organelles, it can be ordered using function as an organising principle. The components that regulate endolysosomal proteolysis may be arranged into a hierarchical model. At the base of the hierarchy are the endolysosomal hydrolases (and substrates) themselves. The activity of these hydrolases is regulated by the luminal conditions within the organelles. The fusion of these organelles may be in turn regulated by Rab proteins. These proteins, and organelle formation, may be regulated by cell signalling pathways, and these pathways may be regulated at the cellular level. Cells are regulated at the tissue level, and so forth. It appears that when the organisation of the endosomal system is viewed in terms of function, these components may be arranged into a hierarchical model. This model consists of tiers, the lowest being the endolysosomal hydrolases, which are regulated by tiers above them.

Implicit in this model is that functional control for a tier is determined by the tier above. In essence, this model describes hierarchical function and co-ordination, with the enzymes themselves being viewed as downstream effectors. The model applies to a cell that is part of a tissue or 'fixed'. For non-fixed cells, like macrophages and neutrophils, the organisational tier above the cellular level may be signals related to its function. In these cells, co-ordinated proteolysis, as a function of overall metabolism, is not as important as proteolysis related to their functions in pathogen clearance. In fact, most professional phagocytes are not fixed and are thus not subject to the same hierarchies as other cells. This model is fairly self-evident, and not a radical departure from how the endolysosomal system has been viewed. In this model, however, the endolysosomal system is approached using function as the basic organising principle. This, as will be described below, may have implications for the treatment of diseases involving the endolysosomal system.

The endolysosomal system provides an entry route for mycobacterial pathogens. These exploit phagocytosis, the very process normally used to clear pathogens from the body. As described in Section 2.1, acidification may be essential for generating a competent endolysosomal proteolytic environment. Not only would acidification create the appropriate conditions for proteolysis by lysosomal proteases, but it also appears to be a prerequisite for phagosome-late endosome fusion, and/or possibly phagosome-lysosome fusion. Treatment of J774 macrophages with either chloroquine or bafilomycin A1, leads to

a block in fusion between the late endosome/lysosome and latex bead-loaded phagosomes. In contrast, cathepsin H, which is found predominantly in the early endosome, is delivered to the phagosome at an enhanced rate. This suggests that early endosome-phagosome fusogenicity is not inhibited, and is possibly stimulated by these drugs (Claus *et al.*, 1998). Thus, the progressive maturation of the phagosome is dependent on progressive acidification.

This property of phagosomes is exploited by *Mycobacterium avium* which avoids destruction and survives within the phagosomal/endolysosomal system. When compared to *Leishmania mexicana*-, IgG-coated latex bead- and zymosan phagosomes, *M. avium* phagosomes fail to acidify. The *M. avium* phagosomes remains at about pH 6.3-6.5, which is very close to the pH of the early endosome. The failure of this phagosome to acidify is due to the absence of the v-ATPase. It is not clear whether the v-ATPase is excluded, or whether it is rapidly removed from the phagosome. Studies by Russel *et al.* (1996) further showed that *M. avium* phagosomes, despite not maturing, are still connected to membrane traffic and may behave as an extension of the recycling early endosome.

Another common factor in the maturation of the phagosome and the autophagic vesicle is the apparent requirement for LAMP proteins. LAMP incorporation appears to occur relatively early in phagosome formation, LAMP-2 being incorporated into latex bead compartments within at least 30 min in J774 cells (Desjardins *et al.*, 1994). LAMP-2 incorporation into the phagosome increases steadily until about 6-8 h, when it reaches a plateau (Claus *et al.*, 1996). This time frame for incorporation into the phagosome corresponds with the maximum incorporation of seven hydrolases into the phagosome: cathepsin B, cathepsin L, dipeptidyl peptidase II, furin, α-galactosidase, β-glucuronidase and β-hexosaminidase (Claus *et al.*, 1998). The *M. avium* phagosomes show incorporation of LAMP-1 suggesting that LAMP-1 incorporation is independent of the H^+-ATPase (Sturgill-Kozycki *et al.*, 1994).

When exploring how *Mycobacterium bovis* escapes destruction by J774 macrophages, Via *et al.* (1997) showed that the *M. bovis*-containing phagosomes fail to incorporate LAMP-2 even after an extended chase of 168 h. It may be argued that incorporation of LAMPs into the phagosome results from the fusion of the phagosome with the late endosome/lysosome. Indeed, the failure to incorporate LAMP-2 was correlated with the lack of incorporation of Rab 7 into the *M. bovis* phagosome (see Fig. 6, Via *et al.*, 1997). Thus, rather than being a

prerequisite for generating a proteolytic environment, the incorporation of LAMPs into the phagosome may be coincidental. However, given their presumed role in protecting the late endolysosomal/lysosomal membrane, LAMPs may be vital components in the phagosomal proteolytic environment.

Acquisition of Rab proteins by the maturing phagocyte is a biochemical marker of maturation. Rab 5 appears to be recruited to the phagosomal membrane at the earliest stages tested (± 30 min, see Fig. 6, Via *et al.*, 1997; Fig. 5, Jahraus *et al.*, 1998) and it is possible that it may have been recruited at an even earlier stage. Rab 5 regulates one of the fastest endocytic transport routes, from the plasma membrane to the early endosome (Rybin *et al.*, 1996). Apart from facilitating content mixing with the early endocytic apparatus (Mayorga *et al.*, 1991; Pitt *et al.*, 1992; Jahraus *et al.*, 1998), Rab 5 may also ensure the rapid delivery of phagocytosed material to the endocytic hydrolases. Rab 5 appears to be retained on the phagosome for up to 168 h (Via *et al.*, 1997). Given the highly dynamic nature of this cell line's endocytic system (see above), the retention of Rab 5 on the phagosomal membrane may still allow for fusion reactions with the late endocytic structures although early endosome fusion reactions may be favoured.

In latex bead phagosomes, Rab 7 also appears to be recruited to the phagosomal membrane at the earliest time point tested (1 h) (Via *et al.*, 1997). Thus, both Rab 5 and 7 are present on the phagosome. Although Rab 5 is present on all organelles of the endolysosomal system, it is found at highest concentrations on the early endosome, and lowest concentrations on the lysosome (Jahraus *et al.*, 1998). Further, Rab 5 appears to stimulate fusion between the phagosome and endocytic structures until about 4 h (Jahraus *et al.*, 1998). Thus, although Rab 5 does appear to be a lifetime resident of the phagosome, it probably functions in stimulating (more) early endocytic fusion events. Rab 7 may be responsible for stimulating later endocytic fusion events. The temporal control of fusion events by these Rabs is probably not distinct, and may be a fail-safe mechanism against bacterial strategies to escape destruction by the endolysosomal system.

The above model for the function of Rab 5 and Rab 7 work is based on the strategy of *M. bovis* in escaping destruction once phagocytosed. *M. bovis* phagosomes, compared to latex bead phagosomes, fail to recruit Rab 7 even after extended periods. In fact, they appear to accumulate Rab 5 (Via *et al.*, 1997). Given the distribution of Rab 5 on the endocytic organelles (see above) it is probable that fusion reactions with the early

endosomes may occur more frequently than with the later endocytic structures. With age, the phagosome becomes progressively less fusogenic (Jahraus *et al.*, 1998), and thus, *M. bovis* may completely escape destruction. In terms of the hierarchical model proposed these mycobacteria affect the formation of proteolytically competent organelles (second organisational tier).

In the process of trypanosome invasion of mammalian cells, lysosomes are exocytosed to the plasma membrane surface in a manner dependent on an elevated intracellular calcium concentration (Rodríguez *et al.*, 1997). This process, mediated by cAMP (Rodríguez *et al.*, 1999), has been detected in a variety of cell types and is microtubule independent (Rodríguez *et al.*, 1997). In all cases, only about 10-20% of the lysosome population was recruited to the plasma membrane surface (Rodríguez *et al.*, 1997). The ability of the trypanosome to cause lysosomal exocytosis is an example of a disruption at a tier above the organelle level.

The above discussions on pathogen invasion of host cells was not meant to be an exhaustive review of these subjects. What is clear from these discussions is that these bacterial pathogens do not try to directly inhibit the proteases, or any other anti-microbial agents that a cell may possess. Rather, pathogens, like *Mycobacterium tuberculosis* or *Trypanasoma cruzi*, probably interfere with the functioning of the endolysosomal system at the organelle and overall cell tiers. In terms of the hierarchical model, this would be at functional tiers above the enzyme level. This strategy may be more effective than dealing with the individual host proteases.

In tumorigenic cells, the organisation of the hierarchy of a 'fixed' or tissue-type cell may be distorted to resemble that of a non-fixed cell. This may be the mechanism that these cells use to acquire signals for protease secretion. Proteolysis is no longer controlled at a tissue level. Further, the unique metabolic nature (like aerobic glycolysis) and microenvironments around tumour cells may result in relationships within a tier being distorted. Thus, for example, the hypoxic, glucose deficient microenvironment around certain tumours may result in cathepsin B and L secretion (Cuvier *et al.*, 1997).

In terms of the theoretical model proposed, it may be easier to block proteolysis by disrupting an organisational tier above the proteases. For example, cathepsin proteases secreted by tumorgenic cells are believed to play an important role in metastasis (Yan *et al.*,

1998). Instead of inhibiting the proteases, it may be more effective to block secretion of the enzymes in the first place. It is possible that even the molecules used by bacterial cells may be used to block secretion of hydrolases by tumor cells. It is also clear that it may be possible to determine hierarchical organisation levels of bacteria or other pathogens, and to target these. The organisational levels may or may not involve organelles, but perhaps molecules that regulate the effector molecules of the pathogenic response may be attacked.

This theoretical model is not wholly unprecedented and has echoes of a field of study in physics called complex emergent systems. Complex systems deals with the organisation of matter in disequilibrium states. *Chaos theory* became a prominent research field in physics during the 1980s (Bak, 1997). Using simple, though non-linear equations, it was shown that within a number of degrees of freedom, systems begin to show extremely aperiodic behaviour (Sardar and Abrams, 1999). In fact, the behaviour of the system could not be predicted after a certain number of degrees of freedom. An example of this is the equations used to described the gravitational force between two objects in space. If a third object is added to the system, calculation of the forces between these objects becomes impossible (Sadar and Abrams, 1999). This is largely due to the fact that these expressions for gravitational force are non-linear. Chaotic systems are highly complex, i.e. they show variability at all scales. In contrast, stable systems are in dynamic equilibrium (Sardar and Abrams, 1999).

The transition between an equilibrium, or stable state, and a chaotic, disequilibrium state, is where systems develop *self-organising criticality* (SOC) (Bak, 1997). Here, at this transition point, systems begin to show complex, emergent behaviour. Whilst these systems exhibit variability at both large and small scales, they also have curious properties, not found in chaotic systems: they have the capacity to adapt, and have emergent properties with dynamics that sometimes work at scales very different from the rules that form them (Holland, 1995). This is a highly significant property of these systems and may be the physical basis for the hierarchical system proposed for the endolysosomal system. This effect of rules can be described with the following example: quarks combine to yield nucleons which in turn combine to form atoms. This process can be carried from here, atoms $\rightarrow$ molecules $\rightarrow$ proteins $\rightarrow$ systems. This 'inter-locking' organisation of matter is the physical basis for the organisation of matter (Holland, 1995). The rules governing the behaviour of enzyme systems are thus based on the rules governing the formation of

nucleons, and so forth. However, the rules defining an enzyme system are, in a real sense, independent of the rules governing nucleon formation (Holland, 1995). Similarly, although protein structure depends on amino acid interaction(s), the folding of proteins may depend on free energy requirements for the whole molecule and not on component amino acid residues. These rules are termed emergent behaviour.

While it may be debatable as to whether the endolysosomal system shows some of the adaptive properties associated with SOC (Bak, 1997) or complex adaptive systems (Holland, 1995), it can be speculated that the physical organisation of the system, and perhaps other enzyme systems, display emergent behaviour. The rules governing the behaviour of the endolysosomal system as a whole, may be different, but not in opposition, to the rules governing the kinetics of the individual lysosomal hydrolases. Hence, the organisation of the endolysosomal system may appear to be hierarchical. This is significant as the endolysosomal system may then be subject to systemic regulation.

7.5 Protease inhibitors as therapeutic agents

In Chapter 6 the rationale and parameters used in selecting protease inhibitors for therapeutic purposes was discussed. The *in vitro* parameters used for determining whether an inhibitor may show promise *in vivo* are the delay time, and the ratio of inhibitor concentration to the K_i. However, it is also clear that the protease concentration may influence the type of interaction between the inhibitor and protease. Future research could be directed at determining whether this effect is relevant *in vivo*.

Lessons from metabolic control analysis suggest that uncompetitive inhibition may be more efficient than competitive inhibition *in vivo*. This efficiency stems largely from the fact that the effect of uncompetitive inhibitors are actually potentiated by substrate. Competitive inhibitors on the other hand, compete against substrate and have to be present at significantly higher concentrations than the substrate, and/or need to have a greater affinity for the enzyme than the substrate does. However, the effectiveness of competitive inhibitors may be overcome by relatively small changes in substrate concentration (Cornish-Bowden, 1995). This has many implications for the use of competitive inhibitors as therapeutic agents in terms of their cost and pharmokinetic properties. To date, no uncompetitive protease inhibitors have been found. As described by Cornish-Bowden (1995), the efficiency of these inhibitors may be the reason that they have not been selected

for in nature. High throughput inhibitor screens for potential protease inhibitors may be modified to detect for uncompetitive inhibition, or at least to search for inhibitors with a significant uncompetitive component to them.

New approaches to drug design are also being explored. Recently, Eisenthal and Cornish-Bowden (1998) modelled the glycolytic pathway in *Trypanosoma brucei*. Their model revealed that by inhibiting pyruvate transport, metabolites may build-up within *T. brucei*, severely affecting the viability of the organism. This approach is a significant step forward in drug design as a systemic attack on the organism may be more effective than inhibiting a single agent. Zhang and Rathod (2002) have studied the effect of the antifolate drug WR92210 on host and malarial parasite dihydrofolate reductase enzymes. It was found that the host cells are able to overcome inhibition by this drug by virtue of their ability to increase production of dihydrofolate reductase. The malarial parasite, with its simpler organisation, lacks the ability to respond to such changes, and is therefore susceptible to WR92210. In this case, the relative sophistication of the mammalian versus parasite organisation is being exploited. These two are important paradigm shifts in the field of drug design, as they show the operation of therapies at systemic levels. Goldberg (2002) has suggested important lead compounds may be missed using screens that simply do head-to-head comparisons. Rather the effects of potential drug candidates may need to be assessed on proteosome, cellular, or even whole organism levels (Goldberg, 2002).

7.6 Future perspectives

Much of the discussion in this final chapter has focused on the need for integrating knowledge from enzymology and cell biology into a systemic approach. Certainly, much knowledge has been gained on the molecules and some of mechanisms that are part of regulatory and effector arms of the endolysosomal system and other systems. This molecular knowledge is vital, but only an introductory step. Integrating this knowledge into systemic models may require a multi-disciplinary approach with contributions from physics, mathematics, enzymology and cell biology. A more complete understanding of the system would certainly yield deeper insights into the evolution of such systems, and perhaps suggest therapeutic strategies for diseases that are associated with them.

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APPENDIX 1

The programme for calculating the redox potential of cysteine:cystine redox buffers was written in BASIC for Mactintosh. The programme used to calculate the redox potentials for a series of cysteine (0.1-0.5 mM):cystine (0.8 mM) buffers is given below. Other redox potentials were similiarly calculated.

```
REM Nernst Equation 3a (with given cystine and cysteine concs)
REM R = gas constant
REM T = temp degrees centigrade
REM TA = absolute temp
REM pH = pH
REM F = Faraday constant
REM Eo = standard reduction potential of cysteine
REM Cys = concentration of cystine
REM C = half concentration of cysteine
REM N = maxima potential concentration of cystine

R = 8.31: T = 37: N1 = 2: pH = 7: F = 96406!: Eo = -0.22: Cys = 0.8

LPRINT "pH =", pH
LPRINT "Cystine =" Cys
LPRINT "Eo", "[Cysteine]", "Cysteine/cystine"
FOR C = 0.1 TO 0.5 STEP 0.1
TA = T + 273
D = Cys/(C*C)
E = LOG (D)
A = (2.303*R*TA)
B = N1*F
M = (2*C)/Cys
H = (A/B)*E
J = Eo + H
```

```
K = 2*C
L = A/F
P = (pH-7)*-L
Q = J+P
PRINT Q, K, M
LPRINT Q, K, M
NEXT C
END
```

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t-Butanol: nature's gift for protein isolation

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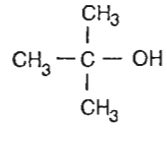
Homogenization in 30% t-butanol is a method with potential for wide application in the isolation of proteins from animal, plant or microbial sources. Unusually, for an organic solvent, t-butanol stabilizes protein structure. It also inhibits enzyme activities and protein/protein interactions. By inhibiting proteolysis and obviating unwanted protein/protein interactions, the presence of approximately 30% t-butanol in a homogenization buffer generally minimizes the formation of artifacts and gives a higher yield. The presence of t-butanol in the homogenate leads naturally on to concentration and fractionation of proteins by three-phase partitioning, simply by the addition of increments of ammonium sulfate. In turn, fractions from three-phase partitioning have a low salt content and are thus poised for ion-exchange chromatography.

Because proteins must be in solution to be separated and purified, protein isolation usually starts with homogenization of the tissue from which the protein is to be isolated.[1] However, the breakdown of cellular compartmentation, concomitant with homogenization, can lead to unwanted protein/protein interactions and the consequent formation of homogenization artifacts. A major problem is proteolysis, caused by the release of proteases from the compartments where they are usually sequestered, and for this reason protease inhibitors are often added to a homogenization buffer. This is less appropriate, however, when cellular proteases themselves are the subject of interest. In the intact cell, proteases and their endogenous inhibitors often occur in different compartments. For example, the 'lysosomal' cysteine proteinases, cathepsins B, L, H, S, etc., occur in the endosomal system whereas stefin B, their endogenous inhibitor, occurs in the cytoplasm. Normally, the cysteine cathepsin/stefin B interaction is a tight, but non-covalent, interaction. However, we have found that formation of an artifactual, covalent, complex of cathepsin L and stefin B is apparently catalysed by a factor concurrently present in liver homogenates.[2,3] The possibility exists that many other similar, artifactual protein/protein interactions may occur in a homogenate and any method that can inhibit both proteolysis and other protein/protein interactions in a homogenate is therefore to be welcomed.

t-Butanol (I) is a reagent used in the established method for protein fractionation known as three-phase partitioning (TPP).[4]

I



In this method, t-butanol is added to about 20–30% (volume ratio) to a protein solution. Subsequent addition of sufficient ammonium sulfate causes the solution to divide into three phases, an upper t-butanol phase (which contains any non-polar solutes), a lower aqueous phase (containing the ammonium sulfate) and a third, intermediate, phase of precipitated (and concentrated) protein. The extent to which protein precipitates into the third phase is a function of the ammonium sulfate concentration and TPP can thus also be used for the fractionation of proteins.

TPP has some advantages over conventional salting out. First, with conventional salting out, the solution becomes increasingly dense with increasing ammonium sulfate concentration, until the stage is reached where it becomes difficult to sediment the precipitated protein. In TPP, however, the precipitated protein floats on the aqueous layer and so, as this becomes ever denser with increasing ammonium sulfate concentration, the precipitated protein floats more and more easily. Secondly, the precipitated protein from conventional salting out generally has a high salt concentration, so salting out must be followed by a desalting step, or by hydrophobic interaction chromatography, which can tolerate high salt concentration. By contrast, the precipitate from TPP has a low salt concentration and so TPP can be followed immediately by ion-exchange chromatography, for example.

Uniquely among common organic solvents, t-butanol stabilizes proteins rather than denaturing them.[4] A common property of all agents that stabilize protein structure is that they are excluded from the protein interior.[5] For example, the sulfate ion, the principal agent in salting out of proteins from water or water/t-butanol co-solvents, stabilizes protein structure

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because the sulfate ion accretes to itself more than 14 water molecules and so the hydrated ion is very large and is unable to access the protein interior.[4] The fact that t-butanol stabilizes rather than denatures proteins may be due to the fact that it too may be too large to gain access to the protein interior. However, most proteins have surface hydrophobic patches, constituting up to 50% of the surface area,[6] and t-butanol appears to bind to these patches, which accounts for the fact that proteins float in TPP.

All enzymes that have been tested are inhibited by t-butanol, with approximately 30% t-butanol being required to completely extinguish activity.[7] In most cases, for instance in all of the many proteins that have been isolated by TPP,[4] this loss of activity may be reversed by removing the t-butanol, for example by dialysis or by phase separation in TPP. The only exception found thus far is haemoglobin, which is denatured by t-butanol, probably by irreversible changes to the inter-subunit interactions. This is not disadvantageous, however, because in many cases haemoglobin is a problematic contaminant in the isolation of proteins from animal tissue. The basis for the inhibiting action of t-butanol is not yet clear, but it has been noted that in the presence of t-butanol some proteins undergo conformational changes, favouring an increased proportion of α-helices.[8] Not only are enzyme activities extinguished by t-butanol, but it appears that protein/protein interactions are also inhibited.[7]

The fact that t-butanol can inhibit proteolysis or enzyme/inhibitor interactions suggests that there may be merit in using t-butanol right at the outset of a protein isolation, that is, the tissue being homogenized in the presence of 30% t-butanol. This concept was tried in the isolation of the lysosomal cysteine proteinase, cathepsin B, from liver tissue, with a resulting 6-fold increase in final yield. A problem attending the isolation from liver of the related lysosomal cysteine proteinase, cathepsin L, is the formation in the homogenate of a proteolytically active, covalent, complex of cathepsin L with the cytoplasmic inhibitor, stefin B, which compromises the yield of the free enzyme.[2,3] Homogenization in 30% t-butanol obviated formation of the complex, resulting in a 40% increase in yield of the free enzyme.[7] With homogenization in 30% t-butanol, the homogenate is poised for fractionation by TPP, simply by adding increments of ammonium sulfate. Since the sulfate ion is more effective at low pH,[4] homogenization in a low p buffer should be the first choice. In tur the low-salt active fraction from TPP poised for fractionation by ion-exchang or affinity chromatography. For the latte removal of residual t-butanol, for exan ple by dialysis, may be advantageous.

It would appear that in the propertie of t-butanol, nature has provided pro teinologists with the gift of a uniquel useful tool. We believe that t-butanol wi have widespread application in the isola tion of proteins from animal, plant anc microbial sources, when its fundamenta difference from other organic solvents such as ethanol and acetone, is fully appreciated.

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In Brief...

Conservation in crisis?

Papers presented at the most recent symposium of the Wildlife Management Association of Southern Africa, held at George, expressed concern about threats to South Africa's biodiversity. It was reported that 'the indigenous freshwater fishes of the Western and Eastern Cape are rapidly approaching widespread and final extinctions'. Dean Impson of Cape Nature Conservation showed that of the 19 indigenous freshwater fishes of the Western Cape rivers (of which 16 are found only there), 11 species are now critically rare or endangered in terms of Red Data Book criteria. Of these fish, only one species was endangered in 1977, three in 1987, nine in 1996 and 11 species in 1999.

Moreover, the provincial conservation agencies have been losing large numbers of staff as long-serving personnel have been given severance packages and then not replaced. In the Western Cape, there were eight staff members involved in freshwater conservation in the provincial conservation authority in 1993. By last year this number had decreased to two. Kas Hamman, the director of Cape Nature Conservation, reported that there had been a 30% loss of conservation staff from the agency in the last three years. At the same time, funding for nature conservation management and research had been reduced throughout the country, it was said. A common sentiment at the symposium was that if current trends in staffing and funding cuts continued, the likelihood of South Africa maintaining its natural resource base was 'minimal'.

Bleek Collection honoured by UNESCO

The remarkable archive of documents recording the language and folklore of a group of San (Bushman) people, held jointly by the University of Cape Town and the National Library, has been formally listed in UNESCO's Memory of the World Register. The papers, compiled over many years by Dr Wilhelm Bleek, his daughter Dorothea and sister-in-law Lucy Lloyd, starting in Cape Town in the 1870s, include nearly 12 000 pages of verbatim accounts of Bushman life, ritual and myth. Bleek was a German linguist who worked originally with /Xam convicts who had been sent to work on a breakwater in Table Bay. The /Xam language is now extinct. These records played an important part in revealing the meaning of San rock art as pioneered by David Lewis-Williams at the University of the Witwatersrand.

The Memory of the World programme, of which the register is an integral part, is to 'guard against collective amnesia', and aims to preserve valuable archives throughout the world.

Healthy funding for sports science

Discovery Health will donate millions of rands for sports science research at the University of Cape Town over the next 10 years. The healthcare company's generosity will be used variously on behalf of the Bioenergetics of Exercise Research Unit, to fund the chair of acclaimed sports scientist Tim Noakes, and the High Performance Centre at the Sports Science Institute. The investment should eventually be worth well over R20 million.

Professor Noakes was recently honoured as one of the 22 founding members of the International Olympic Committee's new Olympic Academy of Science. Although the role of the academy is still undecided, Noakes considers that it should coordinate research to understand how the human body responds to the demands of exercise.

Recognition for water research

The University of Pretoria has established the African Water Issues Research Unit in the Department of Political Sciences, with Anthony Turton, a political scientist who has specialized in the politics of water in southern Africa, as its head. The unit plans to develop a scientific understanding of the role of water in terms of social and economic stability, and to forge strong international links. More information about AWIRA plans is available on the university's website (http://www.up.ac.za/academic/libarts/polsci/awira) or via e-mail (awira@postino.up.ac.za).

The university has also formed a 'strategic alliance' with the CSIR in water research, one of a number of such initiatives between the two institutions, to promote research and training.

Use of 2-Methylpropan-2-ol to Inhibit Proteolysis and Other Protein Interactions in a Tissue Homogenate: An Illustrative Applicatio to the Extraction of Cathepsins B and L from Liver Tissue

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A typical protein isolation from tissue consists of u stream methods of homogenization/extraction and co centration and crude fractionation, where yield is tl most important criterion, and more discriminating dow stream stages such as chromatography, where purific tion is the more important criterion. Three-phase part tioning (TPP[2]) (using *t*-butanol and ammonium sulfat is an upstream procedure used for concentration an crude fractionation early in a protein isolation procedur It has been applied to the isolation of a number of diffe ent proteins, including cathepsins (1, 2), and the princ ples of the method have been explored (3, 4).

A common property of all agents that stabilize pr tein structure is that they are excluded from the pr tein domain (5). *t*-Butanol is unique among commo water-soluble organic solvents in that it tends to n denature proteins, suggesting that it too may be to large to gain access to the protein interior (4). How ever, at a concentration of ca. 30%, *t*-butanol appear to generally inhibit the activity of enzymes (Fig. 1 an Ref. 6). In the case of cathepsins B and L, and presum ably all proteins which have been isolated by TPP (se Table 1, Ref. 4), these changes are reversible and ac tivity is restored upon removal of the *t*-butanol.

Homogenization is often the first step in a protei isolation and many unwanted molecular interaction may occur in a homogenate, e.g., proteolysis (e.g., Ref. 7 or proteinase/inhibitor interactions (e.g., Ref. 8), leadin to the generation of artifacts and perhaps compromisin yield. In previous cathepsin isolations, we have used a sequence of homogenization → pH 4.2 precipitation → TPP. Because *t*-butanol reversibly inactivates many en zymes, including proteases (Fig. 1), it was considered that these three steps may be profitably combined, by incorporating 30% *t*-butanol directly in a low-pH homog

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[2] Abbreviations used: *t*-butanol, 2-methylpropan-2-ol; TPP, three-phase partitioning; Cbz, carbobenzoxy; AMC, 7-amido-4-methyl-coumarin.

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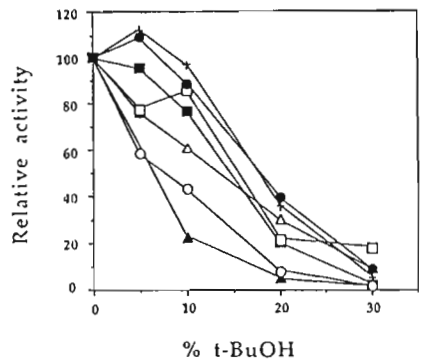

FIG. 1. Effect of *t*-butanol concentration on the activity of some enzymes: (▲) cathepsin B; (○) cathepsin L; (□) cathepsin D; (■) α-glucosidase; (△) fruit bromelain; (●) trypsin; (+) chymotrypsin.

enization buffer. Here we report on the application of this approach to the isolation of cathepsins B and L from rabbit liver and sheep liver, respectively.

Cathepsins B and L were extracted from liver tissue by established methods (8), which served as controls, and by a method incorporating *t*-butanol in the homogenization buffer. Fresh livers from each species were separately diced and separated into two portions, containing equal amounts from each liver, which were separately frozen at −70°C and stored for at least 48 h but not more than 2 weeks.

In the control method for cathepsin B, thawed rabbit liver (originally 300 g, i.e., before being frozen) was homogenized for 3 min in two parts of homogenization buffer (150 mM Na acetate, 1 mM EDTA, pH 4.0) in a Waring Blendor and centrifuged (9000*g*, 20 min, 4°C). The supernatant was adjusted to pH 4.2 with dilute acetic acid and centrifuged (15,100*g*, 20 min, 4°C). *t*-Butanol was added to constitute 30% of the total combined volume of *t*-butanol plus supernatant and a 20–40% ammonium sulfate TPP cut was taken, with centrifuging (15,100*g*, 10 min, 4°C), after each precipitation. The 20% precipitate was discarded and the 20–40% precipitate was redissolved, in one-tenth of the volume of the acid supernatant, in Buffer A (20 mM Na acetate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.0). The redissolved solution was centrifuged (15,100*g*, 10 min, 4°C) to remove any insoluble material. The clarified solution was applied to a column of S-Sepharose (2.5 × 18.3 cm) and unbound material was eluted with Buffer A. Bound material was eluted with a gradient of 0–300 mM NaCl in Buffer A in ca. six column volumes. Fractions active against Cbz-Arg-Arg-AMC were pooled.

In the novel method using *t*-butanol in the homogenate, rabbit liver was homogenized as before, but in a mixture of two parts of homogenization buffer and sufficient *t*-butanol to give 30% (v/v) *t*-butanol in the homogenate. The homogenate was centrifuged (9000*g*, 20 min, 4°C), the supernatant adjusted to pH 4.2 with dilute acetic acid, and the mixture again centrifuged (15,100*g*, 20 min, 4°C). A 20–40% ammonium sulfate TPP cut was taken, as before, and chromatography was as described above.

A similar approach was used with cathepsin L, except that the homogenization "buffer" in the control consisted of 2% *n*-butanol–1% NaCl–0.1% EDTA and a 20–45% TPP cut was taken (8). In the novel method, liver was homogenized in a mixture of two parts of homogenization buffer (150 mM Na acetate, 1 mM EDTA, pH 4.0) and sufficient *t*-butanol to give 30% (v/v) *t*-butanol in the homogenate.

The results for cathepsin B (Table 1), which were essentially reproducible in repeat isolations, reveal very much less activity in the homogenate obtained in the presence of *t*-butanol, despite the fact that the assays were done after removal of the *t*-butanol. However, there is a relatively large increase in activity after the TPP step (2.8-fold) in the case of the homog-

TABLE 1

Extraction and Upstream Fractionation of Cathepsin B from 300 g of Rabbit Liver

	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
			Control			
Homogenate (supernatant)	642	20,897	1,362,183	65	(1)	(100)
TPP cut (20–40%)	98	4,763	228,194	479	7.4	16.8
S-Sepharose	21	63	55,020	874	13.4	4.0
			Homogenization in 30% *t*-butanol			
Homogenate (supernatant)	842	6,155	813,322	132	(1)	(100)
TPP cut (20–40%)	78.5	507	639,585	1261	9.6	78.6 *(47)*[a]
S-Sepharose	14.6	93	198,557	2142	16.2	24.4 *(15)*
Improvement			3.6-fold	2.5-fold		

[a] Figures shown in italics represent yield based on the control homogenate without *t*-butanol.

TABLE 2

Extraction and Upstream Fractionation of Cathepsin L from 180 g of Sheep Liver

	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
			Control			
Homogenate (supernatant)	345	4630	12,537	2.7	(1)	(100)
TPP cut (20–45%)	42	181	3,270	18.1	6.7	26.1
S-Sepharose	33	0.8	2,133	2585.5	954.0	17.0
			Homogenization in 30% *t*-butanol			
Homogenate (supernatant)	520	6682	27,981	4.2	(1)	(100)
TPP cut (20–45%)	44	707	3,339	4.7	1.1	6.0 *(13.3)*[a]
S-Sepharose	35	0.9	2,983	3278.0	782.3	10.7 *(23.8)*
Improvement			1.4-fold	1.3-fold		

[a] Figures shown in italics represent yield based on homogenate without *t*-butanol.

enate containing *t*-butanol. The TPP step removes most of the *t*-butanol, as this is salted out to form a separate phase, while selectively fractionating the proteins. It appears that, in the presence of *t*-butanol, cathepsin B is more effectively extracted but that most of its activity is latent until made patent by the TPP step; by contrast, in the control isolation, there is a relatively large loss of activity during TPP. Here we have reported only on a novel approach to the *extraction* of cathepsin B from liver: for final isolation of the enzyme, a choice of downstream methods is available, including affinity chromatography on Sepharose-Ahx-Gly-Phe-Gly-Sc (9).

In the case of cathepsin L, higher apparent yields were obtained at all steps in the extraction using 30% *t*-butanol, with an increase in yield of about 1.4-fold being finally obtained after S-Sepharose chromatography (Table 2). In previous isolations of cathepsin L from sheep (8), human, and baboon liver (10), we have consistently obtained the enzyme in two forms: as the ca. 24-kDa free enzyme and as a ca. 35-kDa, proteolytically active, covalent complex of cathepsin L with stefin B. In the present study, after homogenization in 30% *t*-butanol, no evidence of this complex was found, supporting the contention that it may be an isolation artifact. Presumably, its formation in the homogenate is inhibited by 30% *t*-butanol.

Although *t*-butanol is not generally denaturing, it does irreversibly denature hemoglobin. This has been used to advantage (e.g., see Refs. 1, 11) but the denaturing effect on hemoglobin does not apply to all proteins with a quaternary structure (11). In general, it appears that homogenization in 30% *t*-butanol gives a "cleaner" extract which is easier to handle in centrifugation steps. This may reflect the fact that *t*-butanol denatures hemoglobin and solubilizes hydrophobic compounds.

We attribute the improved yields of cathepsins B and L partly to the ability of *t*-butanol to inhibit enzyme activity (including proteolysis) and prevent unwanted protein–protein interactions. Homogenization in 30% *t*-butanol is probably not limited in its usefulness only to the isolation of cathepsins as described here and we believe that it may be a generally useful upstream method in the extraction of proteins from animal, plant, and microbial tissues.

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REVIEW ARTICLE

Endolysosomal proteolysis and its regulation

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The endolysosomal system comprises a unique environment for proteolysis, which is regulated in a manner that apparently does not involve protease inhibitors. The system comprises a series of membrane-bound intracellular compartments, within which endocytosed material and redundant cellular components are hydrolysed. Endocytosed material tends to flow vectorially through the system, proceeding through the early endosome, the endosome carrier vesicle, the late endosome and the lysosome. Phagocytosis and autophagy provide alternative entry points into the system. Late endosomes, lysosome/late endosome hybrid organelles, phagosomes and autophagosomes are the principal sites for proteolysis. In each case, hydrolytic competence is due to components of the endolysosomal system, i.e. proteases, lysosome-associated membrane proteins, H^+-ATPases and possibly cysteine transporters. The view is emerging that lysosomes are organelles for the storage of hydrolases, perhaps in an inactivated form. Once a substrate has entered a proteolytically competent environment, the rate-limiting proteolytic steps are probably effected by cysteine endoproteinases. As these are affected by pH and possibly redox potential, they may be regulated by the organelle luminal environment. Regulation is probably also affected, among other factors, by organelle fusion reactions, whereby the meeting of enzyme and substrate may be controlled. Such systems would permit simultaneous regulation of a number of unrelated hydrolases.

Key words: autophagy, endosome, lysosome, proteolysis.

INTRODUCTION

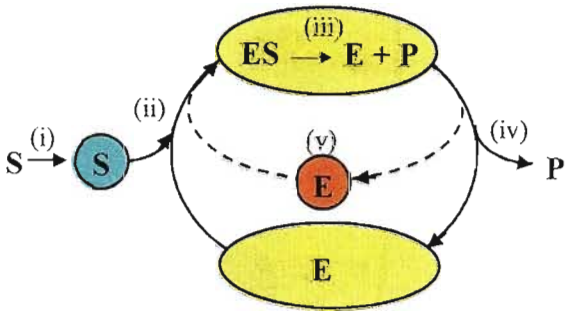
Protein degradation within the cell is principally effected by the endolysosomal, proteosome–ubiquitin and calpain systems. In contrast with the proteosome–ubiquitin and calpain systems, the endolysosomal system largely carries out non-specific bulk proteolysis. The endolysosomal membrane creates a sealed limited environment that allows for optimum functioning of its hydrolases, and yet prevents inappropriate autodegradation. The membrane also houses transporters that remove amino acids, generated by proteolysis, to the cytoplasm.

Apart from degradation, the endolysosomal system is also involved in related functions, such as regulation of signal transduction, antigen presentation and storage. These functions are divided among the various organelles making up the system, which may be thought of as distinct, but connected environments. At the most basic level, the operation of the endolysosomal system of all cells types consists of enzymes that are responsible for degrading substrate that enters the endolysosomal system. The substrate, in turn, must be channelled to the enzymes. Both the enzyme and substrate are maintained in (separate) sealed compartments prior to proteolysis. These sealed environments must be capable of meeting, with the creation of an environment that allows for optimal degradation of the substrate.

Reactions between enzymes (E) and their substrates (S) in solution are described by the reaction (where P is the product)

$$E + S \leftrightarrow ES \rightarrow E + P$$

and can be analysed by conventional kinetic methods. Such enzymes are often regulated by soluble effector molecules. Enzyme reactions in the endolysosomal system, however, can be described by the reactions shown in Scheme 1. As shown, there exists the possibility of regulation at five points: (i) at the point of acquisition of substrate by the endolysosomal system, (ii) at the meeting of E and S by fusion of their respective compartments, (iii) at the establishment of conditions supporting proteolysis (or not) within the (fused) organelle and, possibly (iv) at the point of egress of product from the system. Point (v) represents the possibility of an enzyme being retained in a lysosome for a greater or lesser period (perhaps in an inactive form, but in any event separate from substrate). In terms of flux through the



Scheme 1 Points at which the endolysosomal system may be regulated

Regulation may occur at: (i) the point of acquisition of substrate (S) by the endolysosomal system, (ii) the meeting of enzyme (E) and substrate by fusion of their respective compartments, (iii) the establishment of conditions supporting proteolysis (or not) and, (iv) the point of egress of product from the system. Enzyme may be retained in a lysosome for a greater or lesser period, possibly in an inactive state (v). The colours turquoise, yellow and orange, in that order, represent declining pH.

Abbreviations used: AVd, autophagic vacuole; AV*i*, nascent autophagic vesicle; CHO, Chinese-hamster ovary; ECV, endosome carrier vesicle; ER, endoplasmic reticulum; GILT, γ-interferon-inducible lysosomal thiol reductase; ([^{125}I]tyn-SS-PDL, [^{125}I]iodotyramine linked to poly(D-lysine) by a 3-(propionyldithio)-propionic acid spacer; LAMP, lysosome-associated membrane protein; MPR, mannose-6-phosphate receptors; PDI, protein disulphide isomerase; V-ATPase, vacuolar ATPase.

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system, regulation at (i) and (ii) could be called 'upstream regulation', whereas regulation at (iii), (iv) and (v) could be termed 'downstream regulation'.

Regulation of substrate acquisition into the endolysosomal system and of organelle fusion/budding events [Scheme 1, steps (i) and (ii)] is effected by several signalling networks (reviewed in [1–9]). The signalling networks may include small G-proteins and their signalling cascades, the actin network, and/or phosphatidylinositol 3-phosphate and associated kinases [1–9]. These signalling networks interact with the endolysosomal system through interface molecules that are part of both the endocytosis and the signalling networks [3,7,8]. In the present review, attention will be focused largely on possible regulatory mechanisms operating at points (iii) and (v), i.e. on factors that may affect the activity of the enzymes.

ORGANIZATION OF THE ENDOLYSOSOMAL PATHWAY

The basic organization of the endolysosomal pathway in a mammalian epithelial cell is depicted in Figure 1, although details may vary with cell type. Currently, there are two models to explain how the organelles within the endolysosomal pathway are related to each other [10,11]. Although the two theories differ on the temporal organization of the pathway, the spatial and functional aspects are very similar.

The 'maturation model' suggests that each organelle along the endocytic pathway is a transient, but distinct, compartment that matures into the next organelle along the pathway. In this model, the early endosome is envisioned as being formed *de novo* by the fusion of uncoated primary endosomes derived from the plasma membrane. This transient compartment then matures into a transient late endosome, which in turn matures into a lysosome, the terminal organelle [10]. Each maturation stage has its own unique set of biochemical markers associated with it. These markers and membrane components are recycled by carrier vesicles during maturation [10]. In a related model, proposed by Thilo et al. [12], maturation occurs from the primary endosome until a pre-lysosomal compartment is formed. This compartment then communicates with the lysosome through vesicular traffic.

In the 'pre-existing compartment model', the early and late endosomes are considered to be stable specialized compartments linked by vesicular traffic [11]. The early and late endosomes are regarded as 'compartments', which are stable and do not undergo maturation, but are capable of homotypic fusion. 'Vesicles', unlike compartments, are considered incapable of homotypic fusion. Compartments are considered to be more structurally complex and to have more specialized functions compared with vesicles [11].

The first organelle along the endolysosomal pathway, the early endosome (Figure 1), is a major sorting station. Based on the recycling behaviour of ligand–receptor complexes, the early endosome population may be subdivided into at least two subpopulations, the sorting and recycling endosomes [13]. The luminal pH within the sorting endosome is between pH 6.3–6.5, and is generated by an H^+-ATPase and regulated electrogenically by an Na^+,K^+-ATPase [14,15]. At this slightly acidic pH, ligands, such as low-density lipoprotein and α_2-macroglobulin, dissociate from their cognate receptors. The receptors are recycled to the

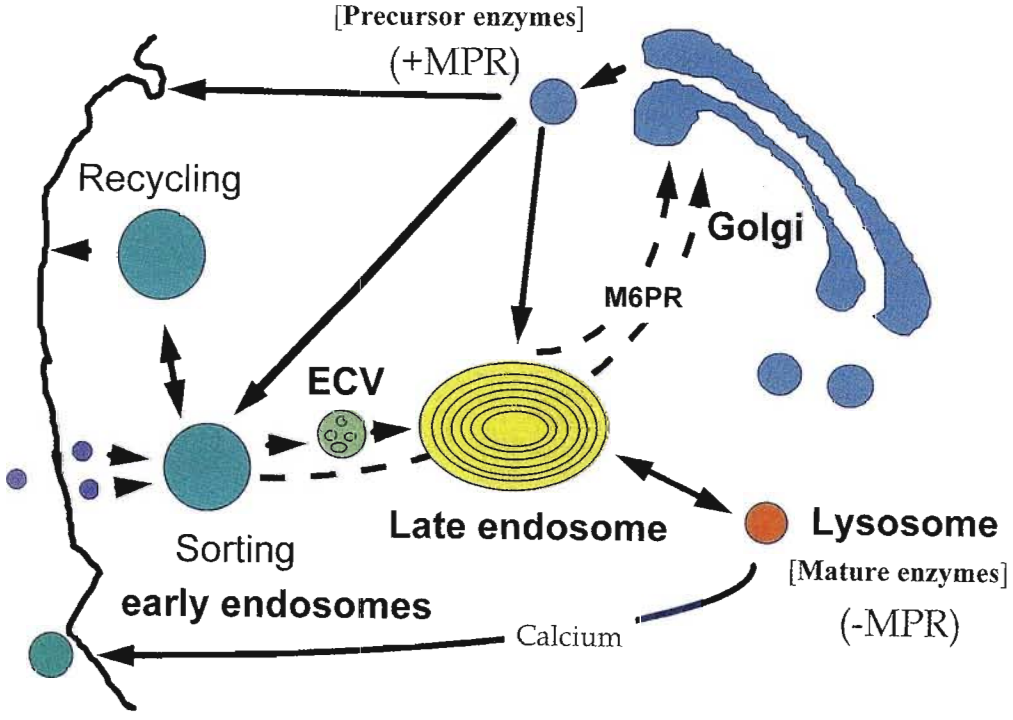


Figure 1 Representation of the endolysosomal system

The major organelles of the endosomal system, the early endosome, late endosome and lysosome, are shown. Solid lines between these organelles may represent vesicular traffic, maturation or direct fusion. Broken lines indicate the recycling of the MPR. Additional components of the endolysosomal system, i.e. phagosomes and autophagic vesicles, are not shown in this simplified diagram, but these organelles interact with the endolysosomal pathway, gaining components that make them proteolytically competent. The colours blue, turquoise, light green, yellow and orange, in that order, represent the declining pH of the intra-luminal conditions. Organelles in different colours may also differ in their redox potential (see the text for details).

plasma membrane, whereas their ligands, low-density lipoprotein and α_2-macroglobulin, are trafficked to the late endosome (reviewed in Mellman [16]). Some receptors, such as the transferrin receptor, can be recycled to the plasma membrane directly from the sorting endosome (fast cycle), or directed to the recycling endosome and then to the plasma membrane (slow cycle) [17]. The recycling endosome may also play a role in directing receptors to the leading edge of migrating cells [17].

The next stage on the endolysosomal pathway is the endosome carrier vesicle (ECV) or multivesicular body. ECVs appear to be large (0.4–0.7 μm in diameter), usually spherical vesicles, often with a membrane-enriched luminal content (reviewed in [11,18,19]), and are responsible for transferring material from early to late endosomes [11].

Formation of ECVs is dependent on the acidification of their luminal interior and can be blocked by the vacuolar ATPase (V-ATPase) inhibitor, bafilomycin A_1 [20]. Indeed, the luminal pH of the ECV drops from that of the early endosome to pH 5.0–5.5 [21]. Endocytosed material may not always be transferred to late endosomes via the ECV. In the mouse macrophage cell line J774, early and late endosomes are capable of fusing directly with each other [22]. This suggests that certain cell types may have specialized endocytic fusion machinery depending on their function.

Distal to the endosome carrier vesicle is the late endosome (sometimes called the pre-lysosomal compartment), followed by the lysosome (Figure 1). A modern definition of a lysosome is that it is 'the terminal organelle on the endocytic pathway and is devoid of recycling receptors' [11]. Complicating the field of endolysosomal proteolysis is the fact that not all authors make a distinction between the late endosome and the lysosome. In the studies of de Duve [23] 'lysosomes' were defined on the basis of two criteria: the existence of a limiting membrane and the presence within the organelle of acid hydrolases. Thus to many authors, lysosomes are any organelles containing acid hydrolases, i.e. 'lysosomal' enzymes (in referring to 'lysosomal' hydrolases here, we put the term 'lysosomal' in quotation marks because some of these enzymes are, in fact, not limited to lysosomes, but are found throughout the endolysosomal system). However, from a modern perspective, this definition encompasses early and late endosomes, lysosomes, phagosomes and autophagosomes. It has also been found that not all 'lysosomal' enzymes have an acidic pH optimum [24,25], and that not all late endosomes/lysosomes are acidic [24].

Both late endosomes and lysosomes contain 'lysosomal' hydrolases and lysosome-associated membrane proteins (LAMPs) [11], and both are enriched in lysobisphosphatidic acid [26]. Lysobisphosphatidic acid is a phospholipid found at high concentrations on the internal membranes of late endosomes, where it may play an important role in the degradation of glycolipids and the transport of membrane proteins and lipids [27]. Under certain conditions, the two organelles may also have similar distributions in density gradients. However, there are differences, for example, mannose-6-phosphate receptors (MPRs) and the regulatory (RII) domain of the cAMP-dependent protein kinase are found on late endosomes, but not lysosomes [11]. Their ultrastructural morphologies are also different. Late endosomes have a complex morphology [11], which is organized by microtubules [28]. In sections, they often have a multivesicular appearance, with intra-luminal membrane whorls (see, for example, [29,30]). In contrast, lysosomes appear as roughly spherical, electron-dense organelles with a simpler organization [11,30]. Based on their relatively simple ultrastructure and their markers, lysosomes may be closer to 'vesicles' than to 'compartments' [11]. A significant difference between lysosomes and other 'vesicles', however, is their capacity to undergo homotypic fusion [31]. To date, no specific marker has been described for lysosomes.

Late endosomes and lysosomes are apparently in dynamic equilibrium. Fluid-phase markers, like BSA-gold, are distributed between the late endosome and lysosome after extended chase periods [11]. Their concentrations of LAMPs are approximately equal, suggesting that their membranes are also in equilibrium. The mechanisms involved in establishing equilibrium may include vesicular transport [32], 'kiss and run' events [33] or direct fusion (reviewed in Luzio et al. [34]) and may vary with cell type. Despite their close association, late endosomes and lysosomes have distinct functional differences. Although containing only 20% of the total hydrolase pool, late endosomes are the main site for proteolysis [29,30]. By contrast, lysosomes contain the bulk of the 'lysosomal' hydrolase pool but only about 20% of total proteolysis takes place in lysosomes. It has consequently been suggested that lysosomes may act as storage organelles for these hydrolases [11,29].

Fusion of late endosomes and lysosomes produces a hybrid organelle with properties of both [34]. Bafilomycin A_1 did not decrease late endosome–lysosome fusion, suggesting that acidification was not a requirement for hybrid formation [35]. However, lysosome recondensation from the hybrid organelle was dependent on a functional V-ATPase [36]. A further requirement for hybrid organelle formation was the presence of intra-organellar Ca^{2+} and calmodulin. Intra-organellar Ca^{2+} is also required for fusion and recondensation of lysosomes to and from the hybrid organelle [36].

Although the lysosome is the terminal organelle of the endolysosomal system, lysosomes should not be viewed as dead-end organelles, as 'secretory lysosomes' may be a feature of many normal cells [37]. An increase in intracellular Ca^{2+} levels typically results in secretion of about 5–15% (rising to 60% in haematopoietic cells) of the total content of lysosomes. A rise in Ca^{2+} levels causes lysosomal membranes to fuse with the cell membrane, resulting in exocytosis of the lysosomal contents [37]. The process is thought to be regulated by synaptotagmin VII [38], and may constitute a mechanism for the repair of damaged plasma membrane [39]. Mechanisms for the secretion of 'lysosomal' proteases are clearly of interest in the context of cancer, where these enzymes have been purported to play an extracellular role. Many cells of haematopoietic origin are capable of exocytosing multivesicular bodies that contain internal vesicles called exosomes. These exosomes may be involved in multiple functions, including T-lymphocyte stimulation (reviewed in Denzer et al. [40]).

DELIVERY OF SUBSTRATES

Substrates destined for degradation can enter the endolysosomal lumen by three broad mechanisms: endocytosis, autophagy and phagocytosis. These mechanisms all result in environments that support proteolysis, using a common set of lysosomal hydrolases. This present review of these processes, although not exhaustive, is aimed at finding common luminal features that may provide insights into the minimal requirements for endolysosomal proteolysis and may also suggest possible modes of regulation of proteolysis.

Endocytosis

Endocytosis can be divided into three distinct mechanisms: receptor-mediated endocytosis, constitutive endocytosis and caveoli formation. Ligand–receptor complexes that enter the endo-

Table 1 Common luminal features of proteolytic organelles of the endolysosomal system

Organelle	Hydrolases	LAMPs	MPR	V-ATPase	Lumenal Ca^{2+}	Cysteine transporter	References
Early endosome	+ (cathepsin H)	−	+	+	+	?	[11,71,72]
Late endosome	+	+	+	+	+	?	[11,71,72,75]
Lysosome	+	+	−	+	+	+	[11,36,71,75,126,127]
Hybrid organelle	+	+	+	?	+	?	[30,34,36]
AVd	+	+	−	+	+	?	[52,60–63]
Phagosome	+	+	−	+	?	?	[11,13,71,72]

lysosomal system can be sorted into one of three pathways: (i) the entire ligand–receptor complex may be recycled back to the plasma membrane; (ii) the ligand–receptor complex may dissociate, with the receptor being recycled and the ligand directed further along the pathway; or (iii) the entire ligand–receptor complex may be targeted to the later stages of the pathway (see Warnock [41] and references therein). This sorting process thus occurs within the early or late endosomes.

In contrast with receptor-mediated endocytosis, substrates entering the pathway by fluid-phase endocytosis are constitutively directed to further stages along the endolysosomal pathway. Pulse-chasing fluid-phase markers, such as horseradish peroxidase and BSA labelled with gold, into a cell first labels early endosomes (5 min), and with longer chase times (< 30 min) the later endosome populations become labelled (see, for example, Aniento et al. [19] and Rabinowitz et al. [42]).

Autophagy

Autophagy is responsible for the destruction of most endogenous proteins, the removal of obsolete and/or damaged organelles, cellular re-modelling during differentiation, metamorphosis and ageing [43,44]. It may be activated when amino acids are limiting *in vitro* [45] and *in vivo* [46]. There are four distinct autophagic mechanisms: macro-autophagy, micro-autophagy, crinophagy and chaperone-mediated autophagy. Micro-autophagy happens when parts of the cytoplasm are taken up directly by lysosomes, occurring when lysosomes invaginate their membranes. Crinophagy is a process whereby secretory granules fuse directly with lysosomes. Chaperone-mediated autophagy effects direct import of cytosolic polypeptides into the endolysosomal system. Micro-autophagy, crinophagy, and chaperone-mediated autophagy have been reviewed previously [44,47,48]. The regulation of autophagy has been reviewed previously [44,49].

Macro-autophagy occurs when entire regions of the cytoplasm are sequestered by a membrane and degraded. This process occurs in at least three distinct stages that are characterized by morphological and biochemical changes to the sequestered cytosol. These stages from sequestration to degradation occur rapidly, with a half-life of approx. 9 min. [50]. The first stage is referred to as sequestration, and is characterized by the formation *de novo* of an organelle referred to as a nascent autophagic vesicle (AV*i*) or the phagophore. A consistent terminology to describe the various stages of autophagy has not yet emerged, and the terminology of Dunn [51,52] will be used here. The signals responsible for the sequestration step are being resolved and may involve G-proteins [49,39–55], a novel protein-conjugation system (see Mizushima et al. [56] and Kirisako et al. [57]), protein and lipid kinases, Ca^{2+} and adenosine nucletotides, and the cytoskeleton (reviewed in Kim and Klionsky [49]).

The source of the sequestering membrane is a matter of dispute, but it may originate from the rough endoplasmic reticulum (ER) [51] or the post-Golgi region [58,59]. AV*i*s have a distinctive structure, with membrane structures and entire organelles engulfed by the forming AV*i* (see, for example Figure 1 in [60]). The AV*i* does not have lysosomal hydrolases or LAMPs associated with it [52,62,63]. It is therefore presumed that the AV*i* is not involved in proteolytic degradation of the engulfed cytoplasm.

The AV*i* apparently undergoes biochemical and morphological changes (maturation), acquiring lysosomal membrane proteins (such as LAMPs) and hydrolases that subsequently allow it to degrade the sequestered substrate [52,62,63]. The AV*i* also develops an acidic luminal interior [52,64] and becomes capable of proteolysis. The fully matured vesicle is called an autophagic vacuole (AVd) [51,52]. In contrast with the AV*i*, the AVd usually has a single membrane, the other membrane structures presumably having been degraded or recycled.

The mechanism of AVd formation from the AV*i* has not been resolved. The AV*i* may fuse directly with lysosomes, which rapidly converts it into an AVd [60]. Alternatively, it has been demonstrated that the endocytic and autophagic pathways converge after the AV*i* and early endosome stages respectively, resulting in formation of amphisomes [61,62]. In either case, the AVd acquires its lysosomal hydrolases from the endolysosomal system.

Phagocytosis

Although a number of cell types are capable of phagocytosis, the most important professional phagocytes are neutrophils and mononuclear phagocytes [65]. The discussion in this review will be limited to macrophages. Macrophages have a complement of cysteine endoproteases similar to that of most epithelial cells. Presumably, the components necessary for a fully functional proteolytic system are the same, or very similar, in both cell types. A notable exception is that macrophages contain the powerful cysteine endoprotease, cathepsin S [66].

Phagocytosed particles are usually in the size range 0.3–0.5 μm in diameter or larger. Their adhesion to the macrophage surface may depend on a number of forces: van der Waal's-, hydrophobic-, electrostatic- or receptor-mediated interactions [65]. The adhesion of a particle to the membrane surface triggers a cascade of signalling events (reviewed in May and Machesky [9], and Kwiatkowska and Sobota [67]) that lead to the formation of a phagosome. Initially, the phagosome lumen resembles the extracellular environment, but the phagosome soon undergoes a series of biochemical changes (maturation), accompanied by changes to the lumen environment. This process is Rab-dependent and involves fusion of the developing phagosome with endocytic organelles [22,68–70]. The changes to the phagosome include: V-ATPase-dependent acidification, and acquisition of Rabs, LAMPs and lysosomal hydrolases [71,72]. Some phagocytosed pathogens escape destruction by disturbing the maturation process. For example, phagosomes that ingest *Mycobacterium avium* cells fail to acidify [73], and those ingesting *M. bovis*

cells fail to incorporate Rab7 [74] and consequently do not participate in late endocytic fusion events [22,74]. Thus the pathogen escapes destruction by the hydrolases that are found in late endosomes/lysosomes.

Based on their importance for endocytosis, autophagy and phagocytosis, the apparent minimal requirements for generating functional proteolytic environments within the endolysosomal system are summarized in Table 1, and are discussed in greater detail below.

COMMON FEATURES OF LUMEN PROTEOLYTIC ENVIRONMENTS

Hydrolases

Most of the soluble endolysosomal hydrolases are synthesized as pre-proenzymes [75]. The signal peptide is cleaved co-translationally and the hydrolases fold into their precursor form within the ER lumen. These precursors undergo asparagine-linked glycosylation and carbohydrate processing, which continues in the Golgi. Within the Golgi, two enzymes, *N*-acetylglucosaminyl-phosphotransferase and α-*N*-acetylglucosaminidase, add a mannose-6-phosphate label [75,76]. Thus the labelled precursors become ligands for the membrane bound MPRs, which direct them to the endolysosomal system (reviewed in Hasilik [75] and Kornfeld [76]). Glycosylation may also serve to protect the hydrolases from destruction within the endolysosomal system [77].

There are over 50 'lysosomal' hydrolases, and some of these enzymes show no sequence homology with each other. Their recognition by the phosphotransferase therefore involves common tertiary structural features [78–80]. There are two MPRs; the 275-kDa cation-independent MPR and the 46-kDa cation-dependent MPR [76]. These receptors have different affinities for the soluble precursors, depending on their carbohydrate structure [81]. Together, these two receptors are able to sort efficiently the diverse array of soluble precursors for targeting to the endolysosomal system. Evidence also exists for MPR-independent targeting mechanisms [75]. Within the endolysosomal system, proteolytic removal of a pro-piece serves to generate the mature active enzyme.

The endolysosomal protease pool may be divided into the endo- and exo-peptidase pools. The endoprotease pool (Table 2) is mainly made up of cysteine and aspartic proteases, and the exopeptidase pool of cysteine and serine proteases. Serine proteases, which are the most numerous and diverse hydrolase class in nature, are notably absent from the endoproteolytic pool. The serine protease cathepsin G is found exclusively in haematopoietic cells and is not a true endolysosomal enzyme (Table 2).

Table 2 Endoproteases found within the endolysosomal system

Name	Catalytic group	M_r	Operating pH*	pI	Distribution	References
Cathepsin B	Cys	27	5–6.5	5.4	Ubiquitous	[66,77,139]
Cathepsin D	Asp	42	2.8–5.0	5.5–6.5	Ubiquitous	[77,144]
Cathepsin E	Asp	100	3–3.5	4.1–4.4	Restricted	[77,145]
Cathepsin G	Ser	30	7.5	10	Neutrophils	[77,138]
Cathepsin H	Cys	28	5.0–6.5	7.1	Ubiquitous	[66,77,143]
Cathepsin L	Cys	29	4.5–6.0	5.8–6.1	Ubiquitous	[66,77,140]
Cathepsin N	Cys	34	3.5	6.2	Ubiquitous	[66,79]
Cathepsin S	Cys	24	5.0–7.5	6.3–6.9	Restricted	[66,79,141]
Cathepsin T	Cys	34	6.9	?	Restricted	[66,77,146]
Cathepsin K	Cys	27–29	6.0–6.5	?	Osteoclasts	[142]
Legumain	Cys	31	4–6	?	Ubiquitous	[66]

* 'Operating pH' is the pH at which the enzyme is stable, which may be different from the pH optimum.

Once a substrate is delivered into a proteolytically competent compartment, it is presumed that the rate-limiting steps in substrate hydrolysis are affected by endoproteases. Their action would generate peptide fragments that would serve as substrates for the lysosomal exopeptidase pool. Thus the present discussion will focus on the endopeptidases, most of which belong to the C1 family of cysteine proteases (Table 2). The C1 family all show a common fold with the archetypal protease of this family, papain. These enzymes are bi-lobular with left and right domains. These domains are highly conserved and all members show common secondary structure elements in their respective domains. Differences between these proteases are usually due to deletions or insertions in the loop regions between the conserved structural elements that comprise the papain fold [66].

The active site is in a deep cleft between the left and right domains. The active site cysteine residue, Cys^{25} (papain numbering), and histidine residue, His^{159} (papain numbering), form a thiolate–imidazolium ion-pair that is responsible for catalysis. Other highly conserved residues are Asn^{175}, which is believed to orientate the imidazolium ring, and Gln^{19}, which is part of the oxyanion hole. Substrates bind into the active site in an extended conformation, and the carbonyl carbon of the scissile bond undergoes nucleophilic attack from the active-site thiol. This results in the release of amine product. The resultant acyl-enzyme reacts with water to release the carboxyl product (deacylation), resulting in the regeneration of the free enzyme. A detailed description of the catalytic mechanism is provided by Storer and Ménard [82].

Why has this family of proteases been selected in preference to other types of proteases? Perhaps uniquely, these enzymes have the following features that make them well suited to the endolysosomal system.

- The nascent enzymes must be capable of being recognized by the phosphotransferase system within the ER. This recognizes structural features, and not a linear sequence [78–80]. This may thus place limits on the hydrolases that could be accommodated within the system. Gene duplication may have created variants of these unique enzymes within the endolysosomal system.
- The lysosomal cysteine proteases are required to cleave a wide variety of substrates and thus their active sites cannot be optimized for a specific substrate. However, the catalytic mechanism of this class of cysteine peptidases is very efficient when compared with the catalytic mechanism employed by serine proteases (see, for example, [83]). This may off-set any loss of efficiency due to a relatively non-specific active site.
- The luminal conditions of pH and redox potential within the late endolysosomal system are designed to denature substrates, allowing for increased hydrolytic efficiency. These conditions may be in a constant state of flux, allowing many different hydrolase classes to operate within the system. The cysteine proteases must be stable to these denaturing conditions and, in addition, must be capable of operating over a broad pH range. The enzymes themselves also have to be fairly resistant to proteolysis. The catalytic ion-pair of the papain superfamily appears to be active over a wide pH range [82], a requirement that cannot be met by any of the other proteolytic classes. This would allow these enzymes to operate in the dynamic endolysosomal proteolytic environment [24]. However, in contrast to other members of the papain superfamily, the endolysosomal enzymes tend to be unstable at neutral to alkaline pH values [66]. This may protect the cell against these enzymes.

The enzymes themselves appear to be stable to the denaturing conditions within the endolysosomal system and have half-lives that run from days to weeks [77].

- Some of the cysteine proteases are restricted to specific organelles. For example, cathepsin H appears to be restricted to the early endosome in J774 macrophages [72]. This suggests that this enzyme must have features that restrict it to this specific organelle, at least in this cell type.
- Finally, endolysosomal proteinases may be capable of regulation without the need for inhibitors. There is no evidence of intra-endolysosomal inhibitors, and yet proteolysis may be shut-down in (storage) lysosomes. This suggests that these enzymes have properties that allow them to be regulated within the endolysosomal system.

Thus quite apart from being efficient hydrolases, these enzymes appear to fulfil a number of requirements that allow them to operate within the endolysosomal environment. Of these additional requirements, the ability to be regulated, without the need for intra-endolysosomal inhibitors, may be amongst the most significant.

Cathepsin D is an aspartic endoprotease found within the endolysosomal system. Unlike its cysteine protease counterparts, this enzyme is capable of operating at very acidic pHs. Thus cathepsin D could effect proteolysis at pH values where the cysteine proteases may be inactivated by protonation of the active-site thiol.

LAMPs

LAMPs are the major protein constituents of late endolysosomal membranes, which is consistent with a putative role in protecting these membranes from hydrolysis and thus preventing leakage of the hydrolases into the cytoplasm. The phagosome, AVd, late endosome and lysosome are all enriched in LAMP proteins. LAMPs are ubiquitously distributed throughout mammalian cells, and make up as much as 50 % of the total protein found on 'lysosomal' membranes. LAMP-1 and LAMP-2 are evolutionarily related: both are type-I membrane proteins, with a short cytoplasmic tail, a transmembrane region and a large luminal domain. The short (10–11 residues) cytoplasmic tail contains targeting information directing the LAMPs to the late endosome/lysosome (reviewed in Peters and von Figura [84]). Proteolytic processing of this tail probably ensures that the protein is retained by these organelles [85]. Although LAMPs and MPRs are directed to the same organelles, they use different targeting machinery [86].

The large luminal domain of the LAMPs has a high carbohydrate content with 16–20 N-linked glycosylation sites, as well as O-linked glycosylation sites. The luminal domain of these proteins also has a proline-rich hinge region and four contiguous disulphide bridges [84]. The high sialic acid content of their carbohydrate moieties contributes to their low pI, which may be of functional significance. LAMPs may participate in aggregation of the soluble lysosomal contents [87]. This could be due to the low pI of the LAMPs, allowing these proteins to behave as cation exchangers. This putative regulatory mechanism will be discussed in greater detail below. When expressed on the cell surface, LAMPs may also play a role in cell adhesion processes [88].

LAMP-1- [89] and LAMP-2-deficient [90] mice have been generated recently. The LAMP-1-deficient mice were viable and fertile, and had lysosomes with properties similar to control lysosomes. The loss of LAMP-1 appeared to be compensated for by increased expression of LAMP-2 [89]. LAMP-2 mutants, on the other hand, showed increased mortality compared with control mice, and showed extensive accumulation of AV*i*s. These results suggest that LAMP-2 is necessary for maturation of the AV*i* to the AVd [90]. The LAMP-1 and LAMP-2 double mutation is lethal [89].

Cysteine transporter

The primary function of endolysosomal proteolysis is to degrade macromolecules for recycling into anabolic reactions. Egress of the products of proteolysis from this sealed environment is largely undertaken by transporters, which have been described for carbohydrate monomers, nucleosides, amino acids and ions (reviewed in Pisoni and Thoene [91]). Two of the transporters that could be involved in regulating the redox potential of the endolysosomal environment are the cystine- and cysteine-specific transporters, and the possible role of these in supporting lysosomal proteolysis will be the focus here.

Disulphide bridges are important in the structural stability of proteins, and reduction of these in the endolysosomal environment may increase the rate of proteolysis. Cysteine is believed to be the physiological reducing agent involved [91]. However, reduction of disulphide bridges by cysteine results in the generation of cystine [92], which is poorly soluble and crystallizes unless it is removed from the 'lysosome'. This occurs in the disease, cystinosis, due to a defect in the cystine transporter [91]. The properties of the cystine transporter have been evaluated *in vitro* using counter-transport and *trans*-stimulation studies, although such studies are complicated by the heterogeneity of 'lysosomal' preparations.

In contrast with other 'lysosomal' transporters, the cysteine-transporter produces a net influx of cysteine *into* 'lysosomes'. When human fibroblasts were incubated with [^{35}S]cystine, at least 50–60 % of the total radioactivity taken up by the cells was found to be associated with 'lysosomes' in the form of cysteine [93]. This uptake occurred rapidly (2–5 min) and the transporter was found to be highly specific. Transport into the 'lysosome' was stimulated when the pH outside was greater than the luminal pH [94]. Cysteine-transport activity has also been described in lysosomal fractions from macrophage and B-cell lymphoma cell lines [95]. In the B-cell lymphoma cell line, cysteine transport activity was also detected in antigen processing compartments [95], suggesting that reduction may be a feature of antigen processing.

The 'lysosomal' cysteine transporter, similar to most endolysosomal transporters, has not been isolated and therefore its intracellular location has not been directly established. However, there is evidence that disulphide reduction occurs in the late endosome compartments, rather than in early endosomes [96,97], suggesting that the cysteine transporter may not be present in early endosomes.

The presence of the cysteine transporter can be interpreted as evidence of reducing conditions within the endolysosomal environment. Indeed, the proteolytic environment may be reducing, but the situation is not as simple as often presented. That reduction does occur is evident from studies on toxins, such as diphtheria toxin, which requires reducing conditions to be active [98,99]. Reduction also appears to be an essential component of antigen processing (see, for example, Collins et al. [97] and Merkel et al. [100]). Presentation of the insulin A chain requires that the cysteine residues are in the thiolate form [101]. Furthermore, Gainey et al. [95] demonstrated that presentation of antigens with disulphide bonds requires compartments capable of cysteine transport. The effect may also be a general one, i.e. that reducing conditions enhance substrate proteolysis, which is not limited to antigen processing.

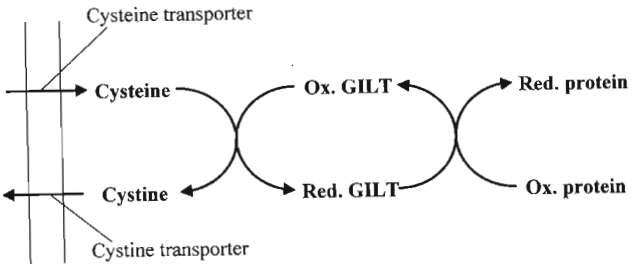
Reducing conditions may affect both proteases and their substrates. It is often assumed that a reducing environment is necessary for the activity of the lysosomal cysteine proteases. However, although isolated cysteine proteases do require reducing agents in order to be active, there is no evidence that these enzymes are oxidized *in vivo* [102–104]. Oxidation may, therefore, be an isolation artefact. In the reduced activated form, the isolated enzymes are more stable to neutral pH conditions [25]. As will be described below, the late endolysosomal environment may not always be acidic and may cycle in neutral or even alkaline conditions. A reducing environment may serve to stabilize these proteases during the purported pH changes.

Reducing conditions would also affect the substrate proteins. It was found that substrates with disulphide links are cleaved with greater efficiency by lysosomal proteases in the presence of a reducing agent [105,106]. Reducing agents are believed to work synergistically with the proteases by reducing the disulphide links, relaxing the substrate structure and exposing more sites for proteolysis. Thus a reducing endolysosomal environment does seem to confer several advantages for proteolysis. However, Lloyd [92] has pointed out that the stoichiometry of the reduction reaction does not necessitate the continuous influx of cysteine and has suggested that the cysteine transporter may simply serve an anaplerotic function, replenishing cysteine lost to autooxidation. It must also be questioned how endolysosomal proteins containing essential disulphide bridges (e.g. cathepsins and LAMPs) are protected against reduction.

A further question is the effect of (low) pH on the reduction potential within the endolysosomal system. Feener et al. [96] constructed a probe comprising [^{125}I]iodotyramine linked to poly(D-lysine) by a 3-(propionyldithio)-propionic acid spacer ([^{125}I]tyn-SS-PDL), which was acid stable and resistant to proteolysis, but sensitive to reduction. This probe was found to be inefficiently reduced within the endolysosomal system, which suggested that the Golgi/*trans*-Golgi may be the main site of reduction [96]. In experiments *in vitro*, the probe was inefficiently reduced by cysteine (5 mM) at low pH [96]. A basis for this result may be found by considering the effect of pH on reduction potential. At fixed cysteine/cystine concentrations, redox potential is inversely proportional to pH, so the reduction potential will be more oxidizing at lower pH. Low pH conditions within the endolysosomal system may thus generate a relatively oxidizing environment.

How may this evidence for and against a reducing endolysosomal proteolytic environment be reconciled? The extremely rapid sequestration of cysteine by 'lysosomes' [93], we believe, is inconsistent with a purely anaplerotic function for the cysteine transporter, as suggested by Lloyd [92]. Moreover, the experiments describing the synergistic effects of cysteine on proteolysis were all undertaken at acidic pH [105,106], suggesting that reduction is not precluded by low pH. To keep the reduction potential more or less constant, however, at low pH, a higher concentration of cysteine and/or a lower concentration of cystine is required. Perhaps the function of the cysteine and cystine transporters is thus to maintain the redox potential within limits by ensuring an influx of the thiol and efflux of the disulphide.

The cytosolic redox buffer is glutathione and cytosolic GSH/GSSG ratios range from 30:1 to 100:1, which corresponds to a redox potential of about −221 mV to −236 mV [107]. Protein disulphide bridges are largely formed in the ER secretory pathway by the enzymes Ero1p (in yeast) and enzymes of the protein disulphide isomerase (PDI) family [108]. The redox buffer in the ER/Golgi secretory pathway may also be glutathione, but here it is more oxidizing than the cytosol, with a GSH/GSSG ratio of 1:1 to 5:1 and a redox potential of about −160 mV to −170 mV [107]. This is in agreement with the optimum GSH/GSSG ratio for the activity of PDI *in vitro*. However, Pdi1p (the yeast analogue of PDI) occurs largely in the disulphide form *in vivo*, and Frand et al. [108] have argued that this balance point is due to efficient oxidation by Ero1p and relatively inefficient reduction by GSH. In the disulphide form, PDI-type enzymes can act as oxidases, transferring disulphides directly to oxidizable proteins.



Scheme 2 Reduction of substrate proteins in the endolysosomal system

It is hypothesized that greater specificity and efficiency of reduction of substrate proteins may be effected by the interposition of GILT-type enzymes between cysteine and the substrate protein. Ox., oxidized; Red., reduced.

An essentially opposite situation may occur in the endolysosomal system where efficient hydrolysis requires the reduction of protein disulphide bridges. Here the reducing agent is thought to be cysteine, but the cysteine/cystine concentration and ratio is unknown. Nevertheless, modelling of the Nernst equation reveals that to achieve a redox potential of −160 mV to −170 mV (i.e. the same as in the ER) at pH 5.0 with a cysteine/cystine redox buffer (at 1 mM total half-cystine), a cysteine/cystine ratio of about 180:1 to 200:1 is required, and to achieve −221 mV to −236 mV (i.e. the same as in the cytosol) requires ratios of 500:1 to 600:1. This could provide a reason for the existence of the cysteine transporter.

A lysosomal reductase, γ-interferon-inducible lysosomal thiol reductase (GILT), has been described [109], which is optimally active at acidic pH, is activated by cysteine (and not by glutathione) and is capable of reducing protein disulphides. Although details are unknown, it is possible that reduction of protein disulphides may occur in a manner such as shown in Scheme 2. The proposed interposition of GILT between cysteine and oxidized proteins (Scheme 2) is analogous to the interposition of PDI between oxidants and protein substrates in the ER, and could play a similar role in introducing greater efficiency and specificity into the reduction reactions. For example, the fact that the [^{125}I]tyn-SS-PDL probe of Feener et al. [96] was not reduced may be because GILT could not recognize this substrate. Similarly, lysosomal proteases with disulphide links may have structural features that exclude them as substrates for GILT.

Acidification

An acidic luminal environment is important for the following functions.

- Receptor-mediated endocytosis and recycling. Receptors, such as the MPR, require acidic conditions in order to discharge their ligands and recycle.
- Movement/maturation of organelles. ECV formation requires a functional V-ATPase [29], which suggests a link between the movement of substrate along the endolysosomal pathway and acidification.

- Activity of membrane transporters. 'Lysosomal' membrane transporters, such as the cystine transporter [110], have acidic pH optima that effectively ensures that amino acid transport is from the lysosome to the cytoplasm [91].
- Activation of lysosomal hydrolases and proteolysis. Many of the lysosomal hydrolases are proteolytically processed to their mature forms under acidic conditions [75], some by auto-processing. Furthermore, many of these hydrolases have acidic pH optima and are therefore maximally active under acidic conditions. An acidic environment may also contribute to denaturation of substrate proteins, which increases the rates of proteolysis.

An acidic luminal environment is established by the V-ATPase and a redox chain [111], both of which pump protons into the lumen. The lysosomal redox chain uses cytoplasmic NADH as an electron donor in a chain that has oxygen as the final electron acceptor [111]. The V-ATPase is made up of two multi-subunit sectors: V_0, an integral membrane sector, and V_1, which is essentially a cytoplasmic sector. The V_0 sector is made up of at least nine subunits (100, 38, 19 and 17 × 6 kDa), and is responsible for proton translocation. The V_1 sector is also made up of at least nine subunits (73 kDa × 3, 58 kDa × 3, 40, 34 and 33 kDa) and is responsible for the ATPase activity. The ATP-binding regions are found on the 73-kDa A-subunits, which show a high degree of co-operativity in ATP catalysis (reviewed in Futai et al. [112]). Also found within the A subunit is a conserved region known as the P-loop. The P-loop has a cysteine residue (Cys^{254}), which is capable of undergoing thiol/disulphide exchange with Cys^{532}, allowing for redox regulation of the V-ATPase [113]. Disulphide-bond formation is believed to induce a conformational change, depressing ATPase activity and inhibiting proton translocation [113]. This mechanism provides a further link, though of a different type, between redox potential and pH.

Although the V-ATPase is responsible for translocating protons into the endolysosomal lumen environment, regulation of the pH of this environment also depends on other factors. Continuous influx of protons into an organelle would result in the generation of a net positive membrane potential that would inhibit further proton translocation. It is believed that inwardly-directed Cl^- ion channels, and perhaps cation channels also, may help regulate the pH of an organelle (reviewed in Futai et al. [112]). The Cl^- channels, in turn, may be regulated by protein kinase A-dependent phosphorylation [114]. As described above, the early endosome pH is regulated by an Na^+/H^+-exchanger that helps to create an internal positive membrane potential, inhibiting further proton uptake [14,15]. The 'lysosomal' pH may be maintained by a Donnan-type equilibrium [116,117]. The number of V-ATPase molecules found on an organelle may also influence the pH of that organelle [115]. Other regulatory features include reversible dissociation–reassembly of the V-ATPase complex, changes in the coupling efficiency between ATP hydrolysis and proton transport and low-molecular-mass activator and inhibitor proteins (reviewed in [115]).

Given the importance of an acidic environment to the proper functioning of the endolysosomal system, are all lysosomes acidic? Some of the cysteine endoproteases found within the endolysosomal system are capable of working over a broad pH range. In fact, cathepsin B has a neutral pH optimum against synthetic substrates *in vitro* [25,118–121]. Cathepsin S is also capable of operating at neutral pH [66]. Butor et al. [24] confirmed the late endolysosomal location of two enzymes, sialic acid-specific 9-*O*-acetyl-esterase and glycosyl-*N*-asparaginase, both of which have neutral to alkaline pH optima, and demonstrated further that not all 'lysosomes' were acidic. They [24] suggested that the 'lysosomal' pH may cycle between acidic and neutral pHs, allowing for the optimal activity of the different hydrolases found within the system. A similar pH cycle may also exist as a consequence of a late endosome/lysosome traffic cycle.

Luminal Ca^{2+}

The role of cytoplasmic Ca^{2+} in lysosomal homotypic fusion [122], lysosome–plasma membrane fusion [37] and phagosome–lysosome fusion [123,124] has been well documented. However, the role of *luminal* Ca^{2+} in regulating proteolysis within these organelles has not been as extensively studied. To the best of our knowledge, the role of intra-organellar Ca^{2+} in regulating phagosome function is unknown. Autophagy, however, appears to depend on intracellularly sequestered Ca^{2+} [125], although the identity of the organelle(s) involved is not known. Lysosomes have been shown to be an intracellular pool for Ca^{2+} [126], and the existence of a lysosomal Ca^{2+} transporter has been demonstrated [127]. The K_m for this lysosomal Ca^{2+} transporter is approx. 5 mM [127], which is significantly higher than the luminal Ca^{2+} concentration [128]. This lysosomal Ca^{2+} transporter may therefore play a role in Ca^{2+} efflux from lysosomes, rather than influx. Calcium sequestration by lysosomes may be via endocytosis, as the extracellular Ca^{2+} concentration can be in the micromolar range [128]. As discussed above, hybrid organelle formation is dependent on Ca^{2+} release from the lumen of late endosomes and lysosomes to facilitate membrane fusion [36]. Luminal Ca^{2+} may also play a role in lysosome reformation from the hybrid organelle [36]. The effect of Ca^{2+} on lysosomal proteases has not, to the best of our knowledge, been reported.

REGULATION OF ENDOLYSOSOMAL PROTEOLYSIS

Regulation by intra-luminal redox conditions?

A conceivable control mechanism for endolysosomal cysteine proteinases is via regulation of the redox conditions in the intra-luminal environment, as discussed above. Both cathepsin B and cathepsin L are stable in the range pH 4.5–6.5 and require a reducing environment for activity *in vitro* [66], which is thought to be required to maintain the active-site cysteine residue in a thiol form. However, there is no evidence that these enzymes are oxidized *in vivo*. Labelling with the inhibitor benzyloxycarbonyl-[^{125}I]iodo-Tyr-Ala-diazomethane (Z-[^{125}I]-Tyr-Ala-CHN_2) of cathepsin B in isolated mouse liver lysosomes did not increase with the addition of dithiothreitol or cysteine [102]. These two reducing agents also did not enhance the activity of cathepsin B in extracts from lung carcinomas and lungs [103,104]. This suggests that redox regulation of the active-site thiol may not occur in the endolysosomal proteolytic environment. Furthermore, it also suggests that these enzymes may not necessarily become oxidized upon their secretion into the extracellular environment.

Although the active site thiols of these enzymes may not be oxidized, it is possible that their ionization state may be altered. At very low pH, the activity of the cathepsins is depressed. This may be related to a pH-dependent change in the ionization state from a thiolate (RS^-) to a thiol (RSH) form. However, at low pH cathepsin D would be active (Table 2).

A study by Krepela et al. [104] suggested that cathepsin B may be inhibited by low concentrations of the thiol (RSH) form of cysteine, which acts as a competitive inhibitor. The inhibitory effect of the thiol form of cysteine could be reversed by increasing concentrations of the thiolate (RS^-) form, and was less prevalent at higher pH. These authors [104] suggested that cysteine may bind into the S1′ subsite of the enzyme, inhibiting its function.

This subsite varies between the different cathepsins [66] and may not be a general regulatory mechanism for all the enzymes. However, since cathepsin B is the most prevalent endoprotease, regulating its function may significantly affect proteolysis within the endolysosomal system.

A study of the effects of changes in redox potential on the activity of (100% active) cathepsin B, showed that the redox potential itself had very little effect on the activity (C. S. Pillay and C. Dennison, unpublished work). It is concluded that, although the endolysosomal lumen may be reducing, changes in cysteine/cystine-induced redox potentials are probably not involved in regulating endolysosomal proteases. Moreover, an expectation of any regulatory mechanism is that it should include all hydrolases within the system, and other endolysosomal hydrolases, e.g. α-glucosidase, have no cysteine residues that could be affected by redox changes.

Unfortunately, the cysteine transporter has not been isolated and its intracellular location, e.g. lysosomes versus late endosomes, and whether it is present in autophagic vacuoles, have not been determined. Thus, at this stage, it is not possible to unequivocally state that reducing conditions are, or are not, a necessary attribute of a proteolytic compartment.

Regulation by pH

Regulation by pH changes, in contrast with redox changes, could simultaneously influence many enzyme classes. Factors that could regulate the luminal pH include: the redox state of the cysteine on the P-loop of the V-ATPase, the presence of active Cl^- transporters on the organelle, a Donnan-type equilibrium, the number of V-ATPases found on that organelle, factors that influence the reversible dissociation/reassembly of the V-ATPase complex, changes in the coupling efficiency between ATP-hydrolysis proton transport and low-molecular-mass activator and inhibitor proteins [112,115], and possibly also a membrane redox chain [111]. What is not clear is how these factors work together to establish the lumen pH. As described above the lumen pH may be dynamic. Within the early endosome, pH appears to regulated by G-proteins (reviewed in Warnock [41]), which respond to transporter entry into the early endosome. Also, Na^+/K^+ exchangers help modulate this environment. The (relatively high) pH within this environment allows cathepsin H to function optimally.

Acidification could also contribute to the storage of endolysosomal enzymes by complexation. Kostoulas et al. [129] found that elastase and other enzymes within the azurophil granules of human neutrophils bound to sulphonated glycosaminoglycans by electrostatic interactions at low pH. It was proposed that this may be a storage mechanism for these enzymes. Pryor et al. [36] suggested that lysosome condensation may be similar to secretory granule formation. A feature of secretory granules is that they contain condensed cores of aggregated proteins, a morphology similar to that of lysosomes [11]. Using bovine pituitary gland cells, it was demonstrated that the granule content proteins and the luminal domains of granule membrane proteins could aggregate at low pH (pH <5.5) [130]. A similar result [130] was obtained for bovine adrenal glands, although aggregation depended on the presence of Ca^{2+}. Proteins destined for constitutive secretion did not aggregate with the granule content and luminal membrane proteins, and this property may serve as a segregation mechanism for those proteins to be stored and those that are constitutively secreted [130].

The aggregation of lysosomal enzymes at pH 4.8 in Chinese-hamster ovary (CHO) cells has been described [131]. This aggregation was disrupted by NaCl, suggesting an electrostatic mechanism. Horseradish peroxidase that had been chased into the CHO cells failed to aggregate with the lysosomal enzymes, suggesting that aggregation was specific. Jadot et al. [87] showed that rat liver lysosomal enzymes aggregated at low pH. This aggregation occurred between pH 4.5 and 5.0, and was mediated by the integral membrane protein LAMP-2. Under these low pH conditions, lysosomal enzymes bind by electrostatic interactions to the LAMP-2 proteins, immobilizing them in a matrix. This process was specific for the 11 lysosomal hydrolases assayed during the experiment; a cytosolic extract and BSA failed to aggregate with LAMP-2 under the conditions tested. Unlike the aggregation observed in CHO cells [131], detergents affected aggregation of the rat liver lysosomal enzymes, suggesting that membrane association with LAMP-2 was vital for aggregation. Lysosomal condensation from the hybrid lysosome–late endosome organelle was shown to be dependent on intra-organellar Ca^{2+} and a functional V-ATPase [36]. If this process involved aggregation of the lysosomal hydrolases, it would be analogous to the pH- and Ca^{2+}-dependent aggregation found in bovine adrenal granules [130]. Furthermore, if this process involved LAMP-2 it may specifically aggregate the lysosomal luminal proteins [87], allowing lysosome condensation without contaminating proteins.

A pH-dependent aggregation mechanism could operate to withdraw lysosomal hydrolases out of the late endosome, and/or it could be a feature of the lysosome itself. An advantage of this complexation mechanism, as opposed to a purported redox-dependent storage mechanism, is that it could encompass several different types of lysosomal hydrolases [87]. Furthermore, as described by Griffiths [11], lysosomes viewed by electron microscopy appear to be small highly dense organelles, a morphology that could be explained by aggregation of their luminal hydrolases.

The concept of lysosomal enzymes aggregating by electrostatic interactions is a fairly old one (see, for example, Henning et al. [132]). However, the acceptance of this as a mechanism for regulating and storing lysosomal hydrolases has been limited. This may be because erroneously low pH optima were assigned to some hydrolases (see Dehrmann et al. [133]), which by chance co-incided with the low pH of lysosomes where the bulk of substrate hydrolysis was thought to occur. Subsequently, it has been found that most proteolysis occurs in late endosomes, which have a pH closer to the revised pH optima of lysosomal proteases. The hypothesis that lysosomal hydrolases may be stored by low pH-induced aggregation does not exclude the possibility that late endosomes may fluctuate between alkaline and acidic pH values, allowing for the activation of different hydrolases with individual pH optima [24]. The lysosome with its lower pH may complex the lysosomal hydrolases, effectively storing them in a precipitated matrix.

This proposed regulatory mechanism could itself be regulated by the factors discussed above that influence V-ATPase activity, i.e. the activity of the lysosomal proteases may be influenced by factors that influence the V-ATPase activity.

CONCLUSIONS AND FUTURE PERSPECTIVES

Complicating the whole field of endolysosomal proteolysis is the lack of a consistent nomenclature, especially concerning the definition of the 'lysosome'. To many authors a 'lysosome' is any organelle containing 'lysosomal' hydrolases. However, we believe that this simple definition has outlived its usefulness and that it may be more usefully replaced by a functional definition, e.g. that lysosomes are organelles mainly for the storage of 'lysosomal' hydrolases (probably in an inactivated state, due to

acid-induced complexation or simply lack of substrate). To become functional, 'lysosomal' hydrolases must be injected into an endosome, phagosome or autophagosome, which are the organelles within which hydrolysis largely occurs. This concept of a lysosome is not too different from the original concept of a 'primary lysosome' [23].

Another modern functional definition of a lysosome is that it is 'the terminal organelle in the endolysosomal pathway and is devoid of recycling receptors' [11]. In the early definition of a 'primary lysosome', this was envisioned as being a vesicle containing newly synthesised enzymes, which budded off the Golgi apparatus [23]. Griffiths [11] has noted that lysosomes are indeed vesicle-sized, so in microscopy studies they may easily be confused with 'lysosomal'-enzyme-carrier vesicles, which have a similar cargo. Unlike carrier vesicles, however, lysosomes are MPR negative.

Many hydrolytic organelles (secondary lysosomes) may be assembled *de novo* when required and this gives an opportunity to determine a minimal requirement for hydrolysis. A common theme applicable to the endolysosomal system, phagosomes and autophagosomes is that enzymes are imported by fusion with lysosomes, acidification is effected by importation of a V-ATPase and egress of products is effected by importation of membrane transporters. Some pathogens exploit the endolysosomal system and escape hydrolysis by blocking the assembly of a complete functional hydrolytic compartment. Of the proposed common factors required to generate a proteolytic luminal environment (Table 1), the least is known about the lysosomal cysteine transporter. Future studies could be directed at determining the location of this transporter within the endolysosomal system, and the role(s) it plays in proteolysis, especially in processes such as phagocytosis and autophagy.

Is endolysosomal proteolysis subject to flux control? With feedback regulation, the downstream rate of proteolysis [Scheme 1, steps (iii) and (iv)] would affect upstream rates of substrate acquisition. Observations by Kominami et al. [134] of the large accumulation of hepatic autophagic vacuoles containing undigested material, after treatment of rats with leupeptin *in vivo*, suggests that there may be no feedback regulation. A second possibility is that proteolysis within the endolysosomal system is sufficiently efficient, and that regardless of the amount of substrate channelled to the proteases, the system could cope. Retention of reserves of temporarily unneeded hydrolases, by storage in lysosomes, would give the system greater elasticity and the ability to respond quickly to changes in demand. In this case, regulation of flux would best be effected in the initial stages of the process, such as substrate acquisition [Scheme 1, steps (i) and (ii)].

It has become apparent that regulation of endocytosis, as an example, is effected by complex signalling networks [1–9]. These signalling networks exert their influence through molecules that interface between the signalling and endocytic pathways. Future research efforts could be directed at attempting to unite the signalling networks, the regulation of endolysosomal fusion reactions and the components that affect the endolysosomal luminal proteolytic environment into an integrated model for proteolysis. Some progress has been made in this regard. Recently, it has been shown that p38 mitogen-activated protein kinase may accelerate the rate of endocytosis in response to oxidative stress [135], providing a direct link between the endocytic rate and the signalling pathways. It has also been shown that the E subunit of the V-ATPase may interact with the Dbl-homology domain of murine Sos1, a guanine nucleotide exchange factor involved in Rac1 activation [136]. Rac1 activation regulates various trafficking events, such as transferrin receptor-mediated clathrin-coated-vesicle formation [137]. Although in this case, the E subunit affected the mSos1–Rac1 signalling pathway [136], it does indicate that there are domains on the V-ATPase capable of interacting with signalling pathways. The activity of Cl^- channels that influence luminal pH, is regulated by protein kinase A-dependent phosphorylation [114]. Thus, it is possible that acidification of endolysosomal organelles may be connected to these signalling pathways. Regulation of conditions within endolysosomal organelles, and in turn the activity of the proteases, may therefore be integrated via signalling networks to endocytosis and changes occurring at the cellular level. In turn, the cell is regulated by the tissue and, ultimately, by the whole-body system. Endolysosomal hydrolases are thus envisioned to be the downstream effectors of a hydrolytic system, which is ultimately regulated at the whole-body level.

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Cathepsin B Stability, But Not Activity, Is Affected in Cysteine:Cystine Redox Buffers

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In order to test the hypothesis that the lysosomal cysteine protease cathepsin B may be redox regulated *in vivo*, cathepsin B activity and stability were measured in cysteine- and/or cystine-containing buffers. Cathepsin B activity in cysteine-containing buffers was similar at pH 6.0 and pH 7.0, over all thiol concentrations tested. In contrast, the stability of the enzyme was greater at pH 6.0 than at pH 7.0. This suggests that the enzyme's operational pH *in vivo* may be < pH 7.0. The activity of the enzyme was depressed in glutathione-containing buffers. When assessed in cysteine:cystine redox buffers (pH 6.0–7.0) cathepsin B was active over a broad redox potential range, suggesting that cathepsin B activity may not be redox regulated. However, at pH 7.0, the stability of cathepsin B decreased with increasing reduction potential and ambient cystine concentration. This suggests that the stability of the enzyme at neutral pH is dependent on redox potential, and on the presence of oxidising agents.

Key words: Cathepsin/Endosomes/Lysosomes/Proteolysis/Redox potential.

Introduction

The late endolysosomal system is a dynamic environment for proteolysis. The pH within late endocytic organelles may fluctuate between neutral and acidic pHs (Butor *et al.*, 1995). Since redox is affected by pH (Segal, 1976), the redox environment may be expected to be similarly dynamic. The redox conditions within the endolysosomal system have, however, not been defined though the luminal environment is believed to be reducing. Reducing conditions would facilitate breakage of disulfide links in substrate proteins, loosening their structure, and increasing rates of proteolysis (Kooistra *et al.*, 1982). It was also speculated that reducing conditions might activate cysteine proteases like cathepsin B. However, it is now known that lysosomal cysteine proteases are active *in vivo* and do not require reduction in order to be active (Wilcox and Mason, 1992; Krepela *et al.*, 1997). Nevertheless, reducing conditions may stabilise these enzymes at neutral pH (Dehrmann *et al.*, 1996).

Reducing redox conditions within the late endolysosomal system are generated by an inwardly directed cysteine transporter (Pisoni *et al.*, 1990). Within the lumen of these organelles cysteine would act on the disulfide links of the substrate, liberating cystine. Cystine, which may also be generated by auto-oxidation, is exported *via* a cystine-specific transporter. A defect in this transporter leads to the disease cystinosis (Jonas *et al.*, 1982; Pisoni and Thoene, 1991).

A report by Krepela *et al.* (1999) suggested that cathepsin B activity may be regulated by the cysteine thiol. Thus, the activity and stability of cathepsin B may be affected by redox conditions, making these conditions potential regulators of the enzyme within the endolysosomal system. Cathepsin B has also been implicated in a number of diseases (Yan *et al.*, 1998), and in apoptosis (Guicciardi *et al.*, 2000; Foghsgaard et al., 2001), where the enzyme is operating in redox environments outside of, and thus different from, the endolysosomal system. In this study, the effect of redox conditions on the activity and stability of cathepsin B were explored, with a view to determine whether the enzyme may be redox regulated *in vivo*.

Results

The Effect of pH and Thiol Concentrations on Cathepsin B Activity

The effect of thiol concentration (provided by cysteine or glutathione) and pH on cathepsin B activity against Cbz-R-R-AMC was explored using Acetate-MES-Tris (AMT) buffers of constant ionic strength (Ellis and Morrison, 1982), with varying concentrations of thiol. In contrast to other reports (see for example Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992; Dehrmann *et al.*, 1996), the activity of cathepsin B at pH 6.0 and pH 7.0, over all thiol concentrations, was similar and was greater than at pH 5.0 (Figure 1). Despite glutathione (GSH) having a lower standard reduction potential than cysteine (Segal, 1976), a GSH-containing buffer of pH 7.0 gave significantly lower enzyme activity over the range of thiol concentrations tested, compared to cysteine-containing AMT buffers at pH 7.0 (Figure 1).

The effect of cysteine on the half-life of cathepsin B at pH 6.0 and 7.0 is shown in Figure 2 (the enzyme was too stable at pH 5 for a half-life to be measured). In contrast to the activity (Figure 1), the half-life was significantly

longer at pH 6.0 than at pH 7.0. The stability of cathepsin B at pH 7.0 in the absence of thiol (AMT redox buffer, pH 7.0, no cysteine) was significantly lower than in the presence of thiol (AMT redox buffers, pH 7.0, 0.2–20 mM cysteine) (data not shown). It appears that cysteine alone increases the stability of cathepsin B at pH 7.0. In contrast, at pH 6.0 cysteine apparently has little effect, as the enzyme was as stable with or without cysteine (Figure 2).

Krepela *et al.* (1999) reported that with increases in cysteine concentration, cathepsin B showed a local minimum in activity. These authors believed that this effect was due to cysteine thiol inhibition of the enzyme. Our data also shows this effect over all pHs tested, and with GSH-containing buffers (Figure 1) as well. Furthermore, our results describing the effect of cysteine on cathepsin B stability (Figure 2) also showed local minima with increases in thiol concentration. These minima corresponded to the minima in the activity data (Figure 1), suggesting a common mechanism that affects both stability and activity.

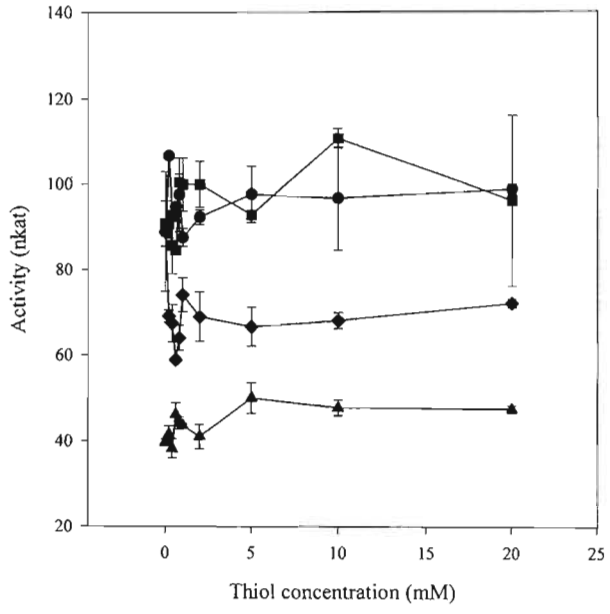

Fig. 1 The Effect of Thiol Concentration on Cathepsin B Activity.
The effect of thiol concentration on cathepsin B activity against Cbz-R-R-AMC was monitored in AMT redox buffers containing cysteine at pH 7 (■), pH 6 (●), pH 5 (▲), and in GSH-containing buffers at pH 7 (◆). The reactions were monitored continuously at 37 °C, and the data represent mean values ± standard deviation (SD) of at least 3 independent determinations.

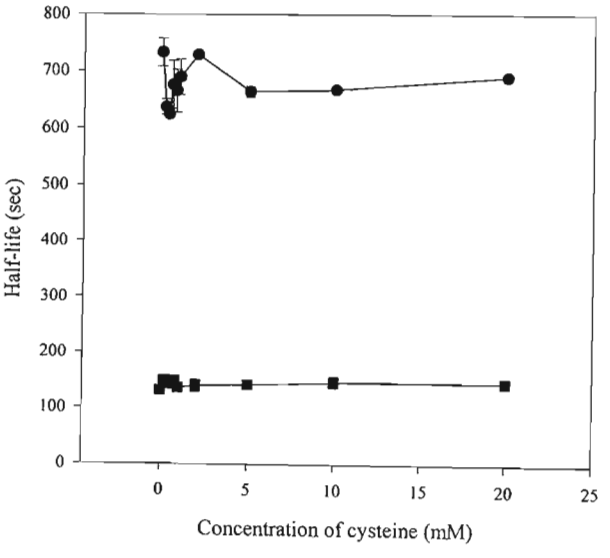

Fig. 2 The Effect of Thiol Concentration on Cathepsin B Stability.
The effect of thiol concentration on cathepsin B stability was monitored in cysteine-containing AMT redox buffers at pH 7 (■) and pH 6 (●). The reactions were monitored continuously at 37 °C, and the data represent the mean ± SD of at least 3 independent determinations.

The Effect of Cystine on Cathepsin B Activity and Stability

Most assays of cysteine protease activity do not include cystine. However, adding cystine to the buffers enabled calculation of reduction potentials using the Nernst equation.

At pH 6.0, starting in a buffer without cysteine, cathepsin B activity increased with decreasing reduction potential, until approximately −80 mV, and thereafter was insensitive to changes in reduction potential (Figure 3A).

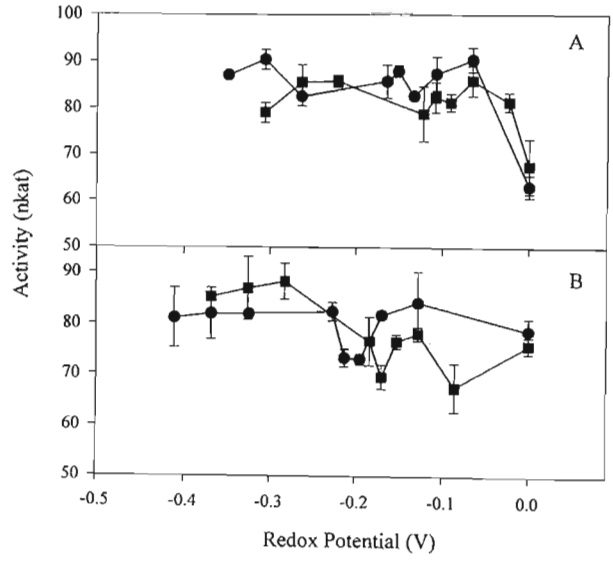

Fig. 3 The Effect of Redox Potential and Cystine Concentration on Cathepsin B Activity at pH 6 (A) and pH 7 (B).
Cathepsin B activity against Cbz-R-R-AMC was assessed with various concentrations of cysteine (0–20 mM) in AMT redox buffers containing cystine at concentrations of 0.2 mM (●) or 0.8 mM (■), respectively. The redox potential of each of the buffers was calculated using the Nernst equation. The data points at 0 mV represent an AMT redox buffer with cystine (0.2–0.8 mM), but no cysteine. The reactions were monitored continuously at 37 °C, and the data represent mean values ± SD of at least 3 independent determinations.

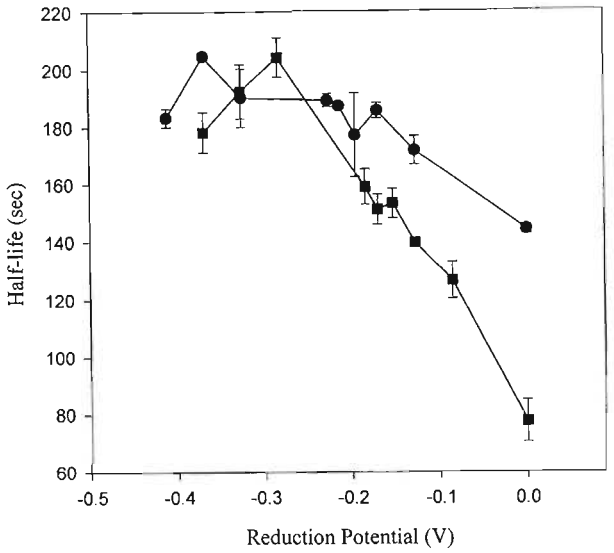

Fig. 4 The Effect of Redox Potential and Cystine Concentration on Cathepsin B Stability at pH 7.
Cathepsin B stability against Cbz-R-R-AMC was assessed in AMT redox buffers containing various concentrations of cysteine (0–20 mM), and 0.2 mM cystine (●) or 0.8 mM cystine (■). The redox potential of each of the buffers was calculated using the Nernst equation. The data points at 0 mV represent an AMT redox buffer with cystine (0.2–0.8 mM), but no cysteine. The data are given as mean ± SD of at least 2 independent determinations.

This suggests that at pH 6.0, cathepsin B is capable of operating over a broad cysteine/cystine generated redox potential range of −80 mV to −350 mV. Similarly, at pH 7.0, the concentration of cystine does not appear to have a significant effect on the activity of cathepsin B (Figure 3B).

As described in the Materials and Methods section, assays were of limited duration to prevent oxidation of redox buffers. The inherent stability of cathepsin B at pH 6.0 made it difficult to assess the effect of cystine on stability at this pH. At pH 7.0, on the other hand, cystine had a marked effect on the stability of cathepsin B. Between buffers with no cysteine and those with reduction potentials of −250 mV, a linear relationship between stability and reduction potential was found (Figure 4), with enzyme stability increasing with decreasing redox potential (*i.e.* increasing concentrations of cysteine). For a given redox potential, stability was reduced in buffers containing 0.8 mM cystine compared to buffers with 0.2 mM cystine. This effect appeared to be dose-dependent with higher stability in buffers with no cystine, followed by buffers with 0.2 mM cystine, and then buffers with 0.8 mM cystine (not shown). However, below −250 mV, with decreasing redox potential (*i.e.* increasing cysteine concentration), the effect of cystine was ameliorated (Figure 4). These results are consistent with cathepsin B undergoing cystine-dependent oxidation, resulting in decreased stability (Figure 4), but in a manner that apparently did not affect the enzyme's activity (Figure 3B).

Discussion

Based on previous work in our laboratory and others, the activity and stability properties of cathepsin B appeared to be disparate. Activity appeared to have a neutral to slightly basic pH optimum, and yet the enzyme was most stable at a more acidic pH. In our assays, however, the activity of cathepsin B was similar at pH 6.0 and pH 7.0. This may be due to differences in the assay and cathepsin B isolation procedures used in these experiments. Isolated cathepsin B is usually not made up of fully active enzyme. Furthermore, the enzyme may be oxidised, and has to be reduced to restore activity. In our assays cathepsin B was affinity purified, and the enzyme was pre-activated before being used in the respective (pH) test buffer. In previous experiments, less than 100% active enzyme may have been used and/or the enzyme was activated in the test buffers themselves. In these cases, differences in activity at different pHs may reflect the degree of reduction at the different pHs. Since reduction occurs more efficiently with increasing pH, the degree of reductive activation, and hence activity, would be expected to be greater at pH 7.0 than at pH 6.0 for a given concentration of thiol. For this reason, perhaps, cathepsin B appeared to have a neutral pH optimum.

In contrast to the activity of the enzyme, the stability of cathepsin B was significantly greater at pH 6.0 than at pH 7.0. This suggests that the enzyme would be capable of operating at maximal activity at pH 6.0, without the loss of stability that occurs at higher pH. Thus, *in vivo*, we believe that it is likely that cathepsin B generally operates at acidic, and not neutral, pHs. The enzyme showed local minima in activity in cysteine (pH 5.0–7.0) and GSH-containing buffers, as well as for stability in cysteine-containing buffers (pH 6.0 and 7.0). We believe that a common mechanism is responsible for these minima, and that cysteine-thiol inhibition of the enzyme (Krepela *et al.*, 1999) does not adequately explain this phenomenon.

GSH is the principal cytosolic reducing agent, with concentrations ranging from 0.1–10 mM (Allen, 1991; Meister, 1995). It appears that the activity of cathepsin B is depressed in GSH-containing buffers, compared to cysteine-containing buffers at pH 7.0. The high cytosolic GSH concentration may thus restrain cathepsin B activity in the cytoplasm. This may provide an additional defence, should cathepsins leak from 'lysosomes' (Brunk *et al.*, 1995).

The effect of cysteine:cystine redox buffers on cathepsin B activity was assessed at pH 6.0 and pH 7.0. In these experiments, it was found that the enzyme activity did not change over a broad redox potential range of −80 mV to −350 mV, *i.e.* from relatively oxidising conditions to relatively highly reducing conditions. The endoplasmic reticulum, for example, is considered to be a relatively oxidising environment, and has an estimated redox potential between −170 and −185 mV (Hwang *et al.*, 1992). In our experiments, cathepsin B was also assayed under relatively highly-reducing conditions (< −250 mV), and the

enzyme activity was not affected. Thus, our data indicates that cathepsin B activity is similar in both relatively oxidising and reducing environments. These results suggest that the activity of cathepsin B is probably not redox regulated *in vivo*.

The stability of cathepsin B was also measured under varying redox conditions. At pH 6.0, the enzyme was essentially stable to all redox conditions tested within the limits of these assays. At pH 7.0, however, it was found that the stability was linearly related to redox potential up to −250 mV (Figure 4). The absolute concentration of cystine affected stability, with higher concentrations of cystine depressing stability at a given redox potential. The negative effect of cystine and oxidising redox conditions on the stability of the enzyme at pH 7.0 could occur through two possible mechanisms. First, cystine and oxidising redox conditions may accelerate the pH-dependent denaturation of the enzyme. Alternatively, cystine-dependent oxidative denaturation of the enzyme may occur more efficiently at pH 7.0 than at pH 6.0, as thiol-disulfide exchange occurs more efficiently at higher pH. These two mechanisms are not mutually exclusive. Thus, despite cathepsin B activity showing no sensitivity to redox potential at pH 7.0, the stability of the enzyme may be affected by redox potential and the ambient concentration of cystine.

In conclusion, our experiments indicate that cathepsin B activity may not be redox regulated. In fact, and in contrast to our initial hypothesis, the enzyme is capable of operating over a broad redox potential. As the endolysosomal redox environment may be highly dynamic, it appears that cathepsin B may be well-suited to the system. Could cathepsin B operate in extra-endosomal redox environments, and would it be stable? We believe that the activity of the enzyme does not preclude it from operating in redox environments other than the endolysosomal system. However, the pH, and the nature and ratios of redox agents present within these environments, may affect the stability of the enzyme.

Materials and Methods

Materials

A Sepharose-Gly-Phe-GlySc affinity matrix for the isolation of cathepsin B was prepared according to the procedure of Rich *et al.* (1986). The Gly-Phe-GlySc ligand was from Bachem (Bubendorf, Switzerland). The S-Sepharose matrix, L-*trans*-epoxy-leucylamido(4-guanidino) butane (E-64), Cbz-R-R-AMC, cystine, dithiothreitol (DTT), GSH and other common reagents were from Sigma-Aldrich (Johannesburg, South Africa). Cysteine (free base) was from Fluka (Sigma-Aldrich, Johannesburg, South Africa). Oxygen-free nitrogen was from Afrox Pty Ltd. (Johannesburg, South Africa). Fresh bovine livers were from Abbakor (Cato Ridge, South Africa).

Isolation of Cathepsin B

Cathepsin B was isolated from bovine liver using the method of Rich *et al.* (1986), with the modification to the homogenisation steps suggested by Dennison *et al.* (2000). Bovine liver was homogenised according to Dennison *et al.* (2000). Three-phase partitioning (Dennison and Lovrien, 1997) was effected on the acid supernatant and the resulting precipitate was dissolved in buffer A (20 mM sodium acetate, 1 mM EDTA, 0.02% NaN_3, pH 5.0) and applied to an S-Sepharose cation-exchanger (2.5 cm × 20 cm). Cathepsin B was eluted using a salt gradient (0–200 mM sodium chloride in buffer A), and dialysed against buffer B (50 mM sodium phosphate, 1 mM EDTA, pH 6.0) The dialysate was applied to a Sepharose-Gly-Phe-GlySc affinity column (1.25 cm × 7 cm) and eluted as described by Rich *et al.* (1986). The eluted fractions were electrophoretically pure (silver stain) and were quantified using the Bradford assay with the modifications suggested by Read and Northcote (1981). Enzyme stocks were periodically tested by E-64 titration (Barrett and Kirschke, 1981) and were typically between 80–100% active. In those fractions where a loss of activity was noticed, the fractions were re-purified by affinity chromatography. It is important that the isolated enzyme is maximally active, as judged by E-64 titration. When cathepsin B is isolated without an active-site-directed affinity chromatography step, only a fraction (usually <40%) of the isolated enzyme is active, with the balance perhaps being partially oxidised to a greater or lesser extent. If less than 100% active enzyme is used, inactivated enzyme molecules may be reactivated by the redox buffers, effectively changing the concentration of active enzyme. For example, Coetzer (1992) found that the proportion of active cathepsin L, measured in an E-64 assay, increased with an increase in DTT concentration in the assay buffer.

Redox Buffers/Activators

In order to study the responses of cathepsin B to changes in redox potential, buffers need to be completely deoxygenated before use, as cysteine is sensitive to oxygenation (Mansoor *et al.*, 1992; Newton and Fahey, 1995) and the redox buffer composition may change without proper protection against oxygen. Assays for activity and stability also have to be of limited duration for the same reason. In our hands, the stability of cathepsin B decreased significantly when the redox buffers were not deoxygenated. Redox potentials were calculated using the Nernst equation (see for example Hwang *et al.*, 1992).

The Effect of pH and Thiol Concentrations on Cathepsin B Activity and Stability

Dehrmann *et al.* (1995, 1996) demonstrated that the activities of cathepsins B and L are sensitive to the ionic strength of buffers used to assay the enzyme. Hence, in this study, AMT buffers, which retain a constant ionic strength over a wide pH range, were employed (Ellis and Morrison, 1982). AMT buffers were used at a final concentration in the assay of 100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA. For those buffers containing cystine, the disulfide was added to the stock AMT buffers: cystine, which is poorly soluble in aqueous solutions, was added after the acetate, and before the other reagents. All buffer solutions were deoxygenated by being boiled, cooled under a stream of oxygen-free nitrogen, and degassed under vacuum. An appropriate concentration of reducing agent was added to accommodate dilution during the assays employed. Further steps were done under a constant stream of oxygen-free nitrogen. Using the method of Gaitonde (1967) it was shown that the thiol concentration within the buffers did not change significantly over the time periods employed in these experiments.

Cathepsin B was pre-activated in assay buffer (0.1 M sodium phosphate, 4 mM EDTA, 2 mM DTT, pH 6) for 10 min, 25 °C. The active enzyme (250 ng, 32 µl) was added to pre-warmed AMT buffer (pH 5–7, 843 µl) containing an appropriate concentration

of thiol (0–20 mm), and Cbz-R-R-AMC (125 µl, 100 µM). The reaction was monitored continuously at 37 °C in a Hitachi F-2000 spectrofluorometer (Advanced Lab Systems, Johannesburg, South Africa). Experiments to determine the half-life of cathepsin B under these redox conditions were conducted as described previously (Dennison *et al.*, 1992; Dehrmann *et al.*, 1995). The enzyme concentration was adjusted in these experiments to ensure that there was a measurable loss in enzyme activity over the assay periods employed (<15 min) (Dennison *et al.*, 1992). Longer assay times were not used as they resulted in appreciable oxidation of the redox buffers. The data represents the mean ± standard error of at least 3 independent determinations.

The Effect of Cystine on Cathepsin B Activity and Stability

Cathepsin B was pre-activated in assay buffer as before. The activated enzyme (250 ng, 32 µl) was added to pre-warmed AMT buffers with or without cystine (0–0.8 mM, 843 µl), containing an appropriate concentration of thiol (0–20 mM), and Cbz-R-R-AMC (100 µM, 125 µl). The reaction was monitored continuously at 37 °C in a Hitachi F-2000 spectrofluorometer. The data represent the mean ± standard error of at least 3 independent determinations. In order to determine the effect that cystine has on the stability of cathepsin B, half-life assays were conducted at pH 7 in AMT buffers with varying concentrations of cystine (0–0.8 mM) and cysteine (0–20 mM). In these experiments at least two independent experiments were conducted, from which the mean and standard errors were calculated.

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