REDOX PROPERTIES OF CATHEPSIN B IN RELATION TO ITS ACTIVITY *IN VIVO*.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg, from January 1997 to December 1998 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 main site for protein degradation along the endosomal pathway is believed to be the late endosome. Lysosomes are thought to be storage organelles that, when necessary, inject proteases into the late endosome. It was hypothesised that differences in the lumenal redox environments between the two organelles could be responsible for their functional differences. In an attempt to quantify this potential difference, the lysosomal cysteine protease cathepsin B was isolated by an improved purification procedure. Several intracellular reducing agents were used to activate cathepsin B, the most effective being cysteine. Cysteine was used to activate cathepsin B under various pH conditions in order to model endosomal conditions. An inverse relationship was found between the pH and the concentration of cysteine required to activate cathepsin B. This suggested that cathepsin B may have an optimal redox potential. In order to determine this potential, cysteine:cystine redox buffers were made up and used in determination of the activity of the enzyme against a synthetic and a whole protein substrate (haemoglobin). No distinct redox potential could be determined using either substrate, but it was found that cystine stimulated proteolysis of haemoglobin. A similar stimulatory effect was observed for cathepsin D and papain hydrolysis of haemoglobin. This effect is possibly due to the ability of cystine to promote substrate structure, effectively increasing the substrate concentration. These findings and other results obtained from the literature have been used to create a model of how proteolysis may be regulated along the endosomal system.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALD	aldolase
AMC	7-amino-4-methylcoumarin
AP-1	Adaptor protein-1 complex
AP-2	Adaptor protein-2 complex
ARF	ADP ribosylation factor
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BSA	bovine serum albumin
C	N,N'-methylenebisacrylamide
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
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino) butane
ECV	Endosome carrier vesicle
EDTA	ethylene diamine tetra acetic acid
Epo	erythropoietin
ER	Endoplasmic Reticulum
GAP	GTPase activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	guanidine diphosphate
GLUT	glucose transporter
GSH	glutathione
GSSG	oxidised glutathione
GTP	guanidine triphosphate

HIF-1	hypoxic inducible factor-1
HRP	horseradish peroxidase
Hsc 73	constitutively expressed heat shock protein of 73 kDa
hsp	heat shock protein
IG	immature granule
LAMPs	lysosomal associated membrane proteins
LDH	lactate dehydrogenase
MPR	mannose 6 phosphate receptor
Mss4	mammalian supressor of sec 4
NADH	beta-nicotinamide adenine dinucleotide (reduced form)
NADPH	beta nicotinamide adenine dinucleotide phosphate (reduced form)
OMP4	hydroxymethylpyridine Ester
PDI	protein disulfide isomerase
PFK	phosphofructokinase
RNAse	ribonuclease A
ROS	reactive oxygen species
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNARE	soluble N-ethylmaleimide sensitive factor receptor
SOD	superoxide dismutase
T	acrylamide
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGN	trans-Golgi network
TH	tyrosine hydroxylase
THI	tetrahedral intermediate
VEGF	vascular endothelial growth factor

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CHAPTER 1

INTRODUCTION

The endosomal system allows a cell to contact and sample the extracellular environment. Depending on the cell type, nutrients, hormones and potential antigens can be proteolytically processed within this system. The system is also responsible for the proteolytic destruction of organelles without threatening destruction of the cell. There are two models to explain the organisation of the endosomal pathway, the maturation model and the pre-existing compartment model.

The maturation model predicts 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 formed *de novo*, by the fusion of many primary endosomes which are derived from the plasma membrane. This transient compartment then matures into a transient late endosome, which in turn matures into a lysosome. 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 (Griffiths, 1996). 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, highly specialised compartments linked by vesicular traffic (Griffiths, 1996). Vesicles, unlike compartments, are incapable of homotypic fusion and have the ability to mature, i.e. to undergo a complex series of biochemical and physical changes with time. A further difference between these organelles is in their structure and function. Compartments tend to be far more structurally complex and have more specialised functions compared to vesicles (Griffiths, 1996). Whilst the two theories differ on the temporal organisation of the pathway, the spatial and functional aspects of the pathway are very similar (Fig. 1). The remainder of this dissertation will describe the endosomal system along the lines of the pre-existing compartment model.

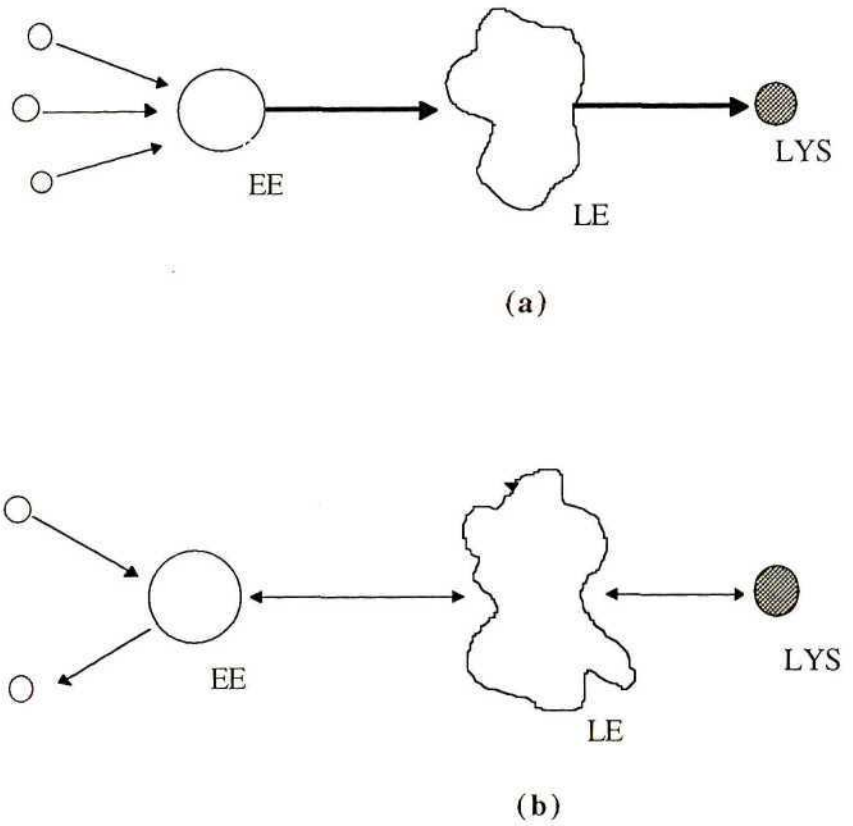

Figure 1. A comparison of the maturation and pre-existing compartment models for the organisation of the endosomal pathway.

(a) Maturation model: In this model primary endosomes fuse forming an early endosome (EE). The early endosome matures into a late endosome (LE), which in turn matures into a lysosome (LYS). Maturation events are indicated by a dark arrow. (b) Pre-existing compartment model: In this model, primary endosomes contact the early endosome (EE) by vesicular traffic. Carrier vesicles from the early endosome carry material to the late endosome (LE), which is linked to the lysosome (LYS) by vesicular traffic.

An extracellular particle may enter the endosomal system through receptor-mediated endocytosis, or through fluid phase endocytosis. The proportion of material taken up through either of these pathways depends on the cell type. Receptor mediated endocytosis is a complex process that occurs with clathrin coated vesicles (CCV's). Upon binding to its cognate ligand, plasma membrane receptors bind specific docking proteins, which in turn recruit adaptor protein 2 (AP-2) complexes. The AP-2 complexes bind to the cytoplasmic tails of the receptors using these docking proteins. The entire assembly of the AP-2 complexes on the plasma membrane is controlled by as yet unidentified GTPases, and possibly kinases and phosphatases (Schmid, 1997).

The AP-2 complexes act as substrates for the binding of cytoplasmic clathrin triskelions. Initially the clathrin assembles in a planar array, but as the clathrin coated pit curves inward during maturation, the clathrin assembly becomes more hexagonal. This rearrangement of the clathrin assembly causes localised distortions in the plasma membrane that are necessary for vesicle formation. Two further steps are needed to drive the formation of the CCV. In the first step, the vesicle develops into a clathrin covered pit attached to the plasma membrane by a narrow neck. The final step requires both ATP and GTP hydrolysis. Whilst the ATPases have not been identified, a 100 kDa GTPase, dynamin, plays a direct role in CCV formation. About 20 dynamin molecules, in their active GTP-bound conformations, self-assemble into a spiral at the neck of the constricted pit. Co-ordinated GTP hydrolysis tightens the spiral at the neck of the pit resulting in vesicle detachment. Recycling of the clathrin is undertaken by hsc 70, a constitutively expressed heat shock protein. Factors required for AP-2 release from the vesicle membrane have not been identified (Schmid, 1997).

Once the CCV loses its coat, it becomes known as a primary endosome. The cargo carried by a primary endosome can follow one of four different pathways: i) the ligand may dissociate from the receptor and follow the pathway to the lysosome, ii) the entire receptor-ligand complex may be directed down the endocytic pathway for destruction, iii) the receptor-ligand complex may be transported to the opposite side of the cell, and iv) the receptor-ligand complex may be recycled back to the plasma membrane (Berg *et al.*, 1995). These membrane fusion events are highly regulated to ensure that vesicles fuse with their target membranes. The selectivity, frequency and the actual fusion events are directed by Rab proteins (Schimmöller *et al.*, 1998).

Rab proteins belong to the Ras superfamily of small GTPases. Rabs are highly conserved and ubiquitously distributed throughout eukaryotic cells. Over 30 Rab isoforms have been described, and these proteins confer specificity to the different intracellular transport routes within the cell. Unique structural elements on the individual Rab proteins allow them to recognise and be localised to their target membranes. Most Rabs are geranylgeranylated allowing them to be membrane associated. However, the Rab proteins will only associate with their target membranes in their active GTP-bound conformations. GTP hydrolysis leads to inactivation of the Rab protein and subsequent loss of membrane association. The Rab cycle is regulated and effected by several proteins such as GDP dissociation inhibitor (GDI), GTPase activating protein (GAP), mammalian suppressor of sec 4 (Mss4) and GDI displacement factor (GDF) (Valentijn and Jamieson, 1998). A further aspect of the Rab GTPase cycle is that each Rab protein hydrolyses GTP at a rate that corresponds to the rate of membrane fusion events directed by that protein (Rybin *et al.*, 1996).

The specificity of membrane fusion events is not directly conferred by Rab proteins, but by the interaction of soluble N-ethylmaleimide sensitive factor receptor (SNARE) complexes. SNARE complexes are formed by the interaction of a vesicle- or v-SNARE, with its cognate t-SNARE found on the target membrane. Genetic experiments have linked Rab function to SNARE interactions, and this link has been described in a recent model by Schimmöller *et al.*, (1998). In this model (Fig. 2) the t-SNARE is protected by a t-SNARE protector that prevents t-SNARE accessibility. Rab proteins in their active GTP-bound conformations could recruit step-specific docking factors to catalyse the deprotection of t-SNARES, allowing for v-SNARE-t-SNARE pairing. Because only active Rab molecules can recruit these docking factors, a further level of specificity is introduced in vesicle targeting.

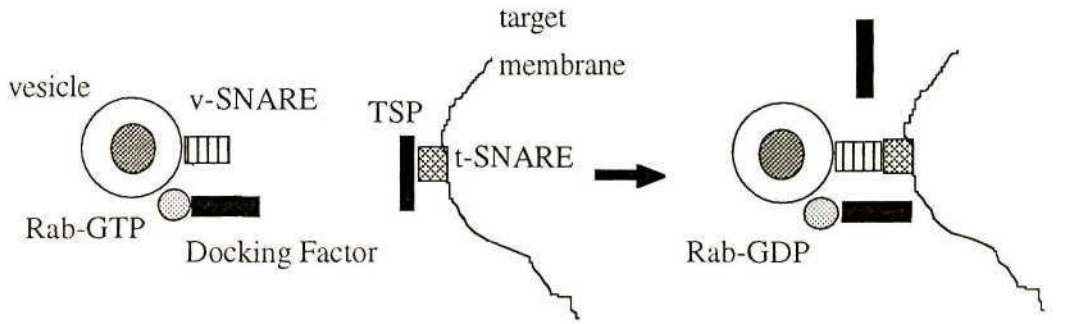

Figure 2. Model for Rab-mediated vesicle docking.

Transport vesicles carry Rabs in their active, GTP bound conformation. These Rabs recruit specific docking factors(s) from the cytosol. The docking-factor Rab complex can recognise a protected t-SNARE, and catalyse the removal of the t-SNARE protector (TSP). This would allow for v- and t- SNARE pairing. Not shown in the figure is the release of the inactive Rab-GDP and docking factor(s) following v-SNARE-t-SNARE pairing (Schimmöller *et al.*, 1998).

Rab proteins undergo a futile cycle of GTP hydrolysis. This GTPase cycle occurs independently of membrane fusion. It is believed that the GTPase cycle of the Rab proteins act as molecular timers for membrane fusion events. For example, Rab 5, which directs one of the fastest intracellular transport routes, has one of the highest GTPase rates for members of the Rab family (Rybin *et al.*, 1996). Homotypic fusion between two organelles may be modulated by this cycle, rather than v-SNARE-t-SNARE interaction (Schimmöller *et al.*, 1998).

The Rab 5-positive early endosome forms the first stage where contents from the primary endosomes are mixed. It is also the stage of the endocytic pathway where proteolytic processing of endocytosed material begins. The proteases responsible for the processing are delivered from the trans-Golgi network (TGN) to the early endosome by mannose-6-phosphate receptors (MPR's) (Ludwig *et al.*, 1991). Newly synthesised lysosomal enzymes

have phosphorylated mannose residues that allow them, at neutral pH, to bind to MPR's in the Golgi. Two types of MPR have been described, the 275 kDa cation independent MPR (CI-MPR) and the 45 kDa cation dependent MPR (CD-MPR) (Berg *et al.*, 1995). Both receptors are transmembrane proteins, and upon binding to a lysosomal enzyme, trigger the recruitment of adapter protein-1 (AP-1) complexes to their cytoplasmic tails. The AP-1 complexes have a low affinity for the MPR cytoplasmic tails and their recruitment is probably mediated through factors such as ADP-ribosylation factor (ARF)-1. In a process analogous to CCV formation at the plasma membrane, clathrin triskelions bind to the AP-1 complexes and are involved in the formation of a coated vesicle (Schmid, 1997). The coated vesicle buds off from the TGN, loses its clathrin coat and fuses with the early endosome (Ludwig *et al.*, 1991). Targeting of the vesicle to a prelysosomal compartment is undertaken by the v-SNARE, syntaxin-6 (Bock *et al.*, 1997).

Lysosomal enzymes that do not bind the MPR's in the TGN may be secreted by default. These enzymes may be recaptured by membrane bound MPR's (Fig. 3). The cytoplasmic tails of the MPR's contain internalisation signals that allow the enzyme-receptor complex to enter the cell by receptor-mediated endocytosis (Johnson and Kornfield, 1992). In regulated secretory cells, which have large granule populations, lysosomal enzyme-MPR complexes may be inadvertently sorted into these exocytic granule populations at the TGN. The maturation of the granules is accompanied by progressive acidification, from the neutral immature granules (IG) to the acidic mature granules (Arvan *et al.*, 1984). Acidification of the granule could trigger both the dissociation of the lysosomal enzyme from the MPR, and the inappropriate activation of the enzyme. Thus, MPR's have to be sorted out of the exocytic pathway at the neutral IG stage. This sorting is accomplished by AP-1 complexes which can bind to MPR's on the IG's and promote the formation of CCV's. These vesicles are then targeted to the endosomal system by syntaxin 6 (Klumperman *et al.*, 1998).

There is accumulating evidence that MPR-independent systems for targeting lysosomal enzymes may also operate in cells. MPR-independent systems have been described for cathepsin B and cathepsin D in rat hepatic cells (Authier *et al.*, 1995), procathepsin L in mouse fibroblasts (McIntyre and Erickson, 1993), and also for procathepsin C in Morris hepatoma 7777 cells which lack the MPR system (Burge *et al.*, 1991). Enzymes may also be delivered to different stages along the endocytic pathway by continuous reticular tubules (Hopkins *et al.*, 1990).

Once the MPR-hydrolase complexes reach the endosomal system, the acidic conditions within the system trigger the release of the enzyme. The MPR's are recycled back to the TGN in a Rab 9 dependent manner (Riederer *et al.*, 1994). The factors required for enzyme dissociation by MPR-independent systems are not known. Proteolytic processing of a

substrate begins in the early endosome with the bulk of substrate hydrolysis being carried out in the later stages of the pathway.

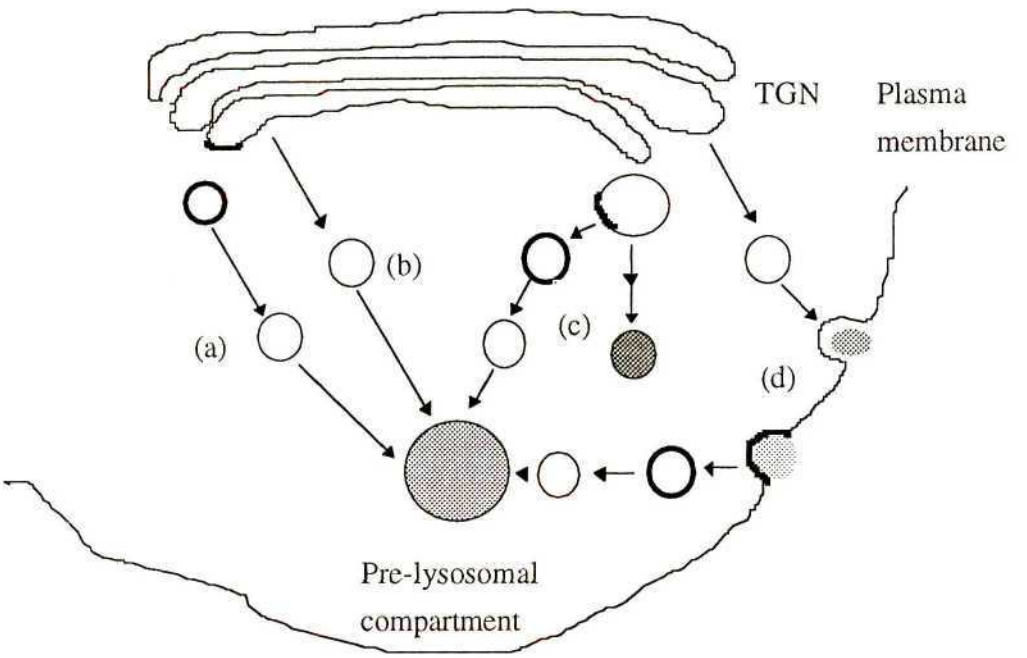

Figure 3. Entry pathways for lysosomal hydrolases into the endosomal system.

Lysosomal hydrolases can enter the endosomal system by one of four pathways. (a) Lysosomal hydrolases can bind to MPR's and leave the TGN by CCV's. These vesicles fuse with a prelysosomal compartment, and the MPR's discharge their cargo under acidic conditions. (b) Lysosomal hydrolases can also enter the endosomal system by as yet uncharacterised transporters. (c) Lysosomal hydrolase-MPR complexes that are inadvertently sorted into secretory granules, can be extracted by CCV formation. (d) Hydrolases that are exported out of the cell can be recaptured by plasma membrane MPR's and routed to the endosomal system by receptor-mediated endocytosis. The presence of clathrin is indicated by dark lines.

The next stage along the endosomal system is the endosomal carrier vesicle (ECV). According to the pre-existing compartment model, ECV's bud off from the early endosome and carry material destined for degradation to the late endosome. ECV's are between 0.3-0.4 μM spherical vesicles that have a distinctive membrane enriched lumenal content. The lumenal pH of the ECV drops from the early endosomal pH of 6.0-6.5 to values of pH 5.0-5.5 as the vesicle matures. The ECV movement between the early and late endosomes is microtubule dependent. The ECV's lack the ability to fuse with one another or with the early

endosomes that they were derived from, but are capable of fusing with late endosomes (Griffiths, 1996).

The ECV fuses with the next stage of the endosomal pathway, the late endosome. The late endosome is morphologically and functionally distinct from the lysosome. The late endosome has a relatively complex organisation and a lighter density on Percoll or sucrose gradients when compared to lysosomes. Lysosomes tend to be smaller, more spherical, organelles of high density. A further difference is that late endosomes have markers like Rab 7, the 2 MPR's and the regulatory (RII) domain of the CAMP-dependent protein kinase, that are not present on lysosomes. Both organelles have about equal concentrations of lysosomal associated membrane proteins (LAMP's), whose function is not yet known (Griffiths, 1996).

Despite these morphological differences, in much of the literature there is a failure to appreciate the distinction, and the functional differences, between these two organelles and the term "lysosomes" is often used loosely. The original definition of lysosomes was that they were acidic organelles, containing acid hydrolases. From a modern perspective, this definition encompasses both lysosomes and late endosomes, though lysosomes may not all be acidic (Butor *et al.*, 1995). A modern definition of lysosomes is that they are terminal vesicles on the endosomal pathway that are relatively dense, and which are devoid of recycling receptors, such as the two MPR's (Griffiths, 1996).

The position of lysosomes on the endosomal pathway, and the absence of receptors are properties most easily determined by microscopical studies, including immunocytochemistry. Density is frequently used in sub-cellular fractionation by centrifugation to isolate lysosomes. Often however, inadequate consideration is given to the contamination of genuine lysosomal fractions by late endosomal fractions (Griffiths, 1996). Great care is therefore necessary when interpreting exactly what any particular author means when using the term lysosome. In those density gradient fractionation experiments where the author(s) have not made the distinction between late endosomes and lysosomes, the term lysosome is placed in inverted commas to indicate this. The hydrolases within the endosomal system, despite not being confined to the lysosome, have retained the prefix "lysosomal", for convenience.

Most (80-90%) of the total lysosomal hydrolase pool resides in the lysosome, with the exception of human fibroblasts where there is an equal division of the enzyme pool between the two organelles (Rome and Crain, 1981). Using an ^{125}I -labelled-tyramine cellobiose-ovalbumin conjugate, whose degradation products remain trapped at their site of formation, it was demonstrated that despite only having 20% of the total lysosomal hydrolase pool, the bulk of proteolysis (80%) appears to occur in the late endosome. It was reasoned that

lysosomes may be storage organelles that when necessary inject part of their hydrolase pool into the late endosome, where the bulk of substrate hydrolysis occurs (Tjelle *et al.*, 1996).

These results suggest that there must be a regulatory mechanism that injects hydrolases into the late endosome when necessary. Secondly, they suggest that the environment within the late endosome must support proteolysis when compared to the lysosomal environment. Clues to the regulatory mechanism that injects proteases into the late endosome may have been described in J774 macrophages in a recent paper by Claus *et al.* (1998). These authors found that subsets of lysosomal proteases were injected into phagosomes, and could be sorted out of them depending on phagosomal pH. The details of such an envisaged regulatory mechanism must await further investigation.

The second question raised by the work of Tjelle *et al.* (1996) is how the environment of the late endosome supports proteolysis. Alternatively, how does the lysosome ensure that proteolysis is 'switched off'. This activation mechanism that distinguishes these two organelles must fulfil 4 requirements:

- It must be able to account for the activation of proteolysis within the late endosome when compared to the lysosome.
- The activation mechanism must be able to operate over a wide range of pH values. Butor *et al.*, (1995) have demonstrated that late endosomes/lysosomes have pH values that fluctuate between pH values less than 4, up to alkaline pH's. These pH conditions are presumably necessary to accommodate the widely different pH optima of the various lysosomal hydrolases.
- The activation mechanism must apply to all the lysosomal hydrolases within the system.
- Ideally, the system should account for the morphological differences between late endosomes and lysosomes.

In the present study, attention was focused on the lysosomal proteases. Lysosomal endoproteases are mainly cysteine proteases, which are functional only under reducing conditions. The crystal structure of the lysosomal cysteine protease cathepsin B showed that the active site thiol residue was in the form a reversible sulfinic acid (Musil *et al.*, 1991). Reducing conditions convert the sulfinic acid into a thiol, and depending on the pH, activate the protease. It was consequently hypothesised that the functional/activational differences between the late endosomes and lysosomes could reflect differences in their intralumenal redox potential, i.e. late endosomes might support proteolysis by maintaining a relatively reducing environment. Conversely, the relatively oxidising conditions within the lysosome might inactivate the cysteine proteases, storing them in a latent form. Thus, proteolysis could be regulated without the need for inhibitors and regulatory systems for these inhibitors.

Besides activating cysteine proteinases, reducing conditions also promote proteolysis by breaking the disulfide bonds of a substrate protein, thus relaxing the substrate structure. This in turn exposes more potential sites for proteolysis. Using the lysosomal enzymes cathepsins B, D, H and L, it was found that reducing conditions and proteolysis act synergistically to degrade a substrate (Kooistra *et al.*, 1982). Disulfide reduction appears to be a pre-requisite for the proteolytic processing of certain antigens. Using the substrates bovine ribonuclease A (RNAse) and hen egg lysosome, it was found that prior reduction and carboxymethylation of these substrates resulted in increased antigen presentation by peritoneal macrophages when compared to the response elicited by native substrates (Collins *et al.*, 1991). In contrast to the results of Kooistra *et al.* (1982), these authors also found that disulfide cleavage of their redox probe, α-2 macroglobulin-bisthiopropionyl-glycyl tyrosine, occurred independently of, and before significant proteolytic degradation (Collins *et al.*, 1991).

The reducing conditions within the "lysosome" are generated by a cysteine-specific transporter. Clues to the existence of the transporter came from uptake studies of ^{35}S-cystine in human fibroblasts. It was found that 50-60% of the total radioactivity was associated with the "lysosomal" compartment in the form of cysteine. Further, this uptake was rapid, occurring within 2-5 min of the pulse (Elferink *et al.*, 1983). Pisoni *et al.* (1990) described the properties of the transporter in human fibroblasts. The transporter displays a K_m of 53 μM for cysteine uptake at pH 7.0 and 37°C. It also exhibits the highest activation energy for any known lysosomal transport system. The transporter is highly specific for cysteine, and displays an optimal pH between 7.2-8.0.

The reduction of a disulfide bridge within a substrate generates the oxidised form of cysteine, cystine (Segal, 1976). A transport system for cystine efflux has also been described. A defect in this system is responsible for the disease cystinosis. Efflux of cystine from "lysosomes" occurs with a half-time of 25-45 min at 37°C and pH 7.0 in human leukocytes, fibroblasts and lymphoblasts. Cystine efflux is stimulated by MgATP and by the polyamines spermidine, putrescine, cadaverine and spermine. Interestingly, human leukocyte lysosomes can take up cystine, with a pH optimum between 5.5-6.5. K_m values of 0.3-0.5 μM for cystine uptake at pH 7.0 and 37°C have been described in human leukocytes and mouse fibroblasts (Pisoni and Thoene, 1991).

Cystine that is transported out of the lysosome is reduced in the cytoplasm. The cytoplasm has a high concentration of the tripeptide, glutathione (GSH). GSH is the principle cytoplasmic reducing agent and is responsible for maintaining a reducing environment in the cytoplasm. GSH reduces cystine to form cysteine and a mixed disulfide of GSH and cysteine. This latter product is further metabolised to generate GSH and cysteine (Meister, 1995).

Meinesz (1996) developed a dual fluorescent labelling system in order to determine whether the endosomal system in the breast cancer cell line MCF-10A, contained lysosomes that were relatively oxidising. The LysoTracker probe was used to label lysosomes, whilst the dye C-2938 was used to determine which organelles were oxidising. However, at the outset of this present study it was found that the C-2938 dye was pH and redox sensitive. Thus, the changes in fluorescence associated with the dye may not reflect changes in redox potential, but changes in pH. Because of the inherent uncertainty associated with using the C-2938 dye, attention was focused on the enzymes themselves. The lysosomal cysteine protease, cathepsin B was chosen for study because a convenient and rapid isolation procedure of this enzyme had been developed in this laboratory. The ability of cathepsin B to be activated by various reducing agents was investigated to determine whether the enzyme could be activated under non-lysosomal conditions. The relationship between pH and reduction potential on cathepsin B activation was also explored to determine possible activation states of the enzyme along the endosomal pathway. Finally, an attempt was made to determine the optimal redox potential of cathepsin B.

CHAPTER 2

MATERIALS AND METHODS

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

2.1 Materials

Most of the common chemicals used during this study were purchased from BDH, Merck or Boehringer Mannheim and were of the highest purity available. Coomassie brilliant blue R-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) and Sephacryl S-100 were from Sigma, St. Louis, MO. The materials for constructing the pepstatin affinity resin, pepstatin A, aminohexylagarose, N-hydroxsuccinimide and dicyclocarbodiimide, as well as the protease substrates CBz-R-R-AMC, CBz-F-R-AMC, haemoglobin and azocasein, were also from Sigma. The cation exchange chromatography gel, S-Sepharose, was from Pharmacia Biotech, Uppulsa, Sweden. The P-60 molecular exclusion gel and the hydroxyapatite medium were from Bio-Rad Laboratories, Richmond, CA. UltraFuge ultrafiltration centrifuge filters were from Micron Separations Inc., Westbro, MA.

2.2 Protein assays

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

2.2.1 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 like 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.

2.2.1.1 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.

2.2.1.2 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.

2.3 Concentration of enzyme samples

Many of the enzyme isolation procedures used during this study required concentration of the dilute protein samples before they could be used. 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 continuos 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 (2 000 x g, 30 min, 4°C). Under these 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.

2.4 Electrophoretic techniques

2.4.1 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).

2.4.1.1 Reagents

Monomer solution [49.5% (m/v) acrylamide (T), 3% (m/v) N,N'-methylene-bisacrylamide (C)]. Acrylamide (48.0 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 100 ml dist.H_2O.

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 1 litre with dist.H_2O and the pH checked.

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 in treatment buffer before electrophoresis.

2.4.1.2 Procedure

The composition of the resolving and stacking gels used to construct the Tris-Tricine system are described in Table 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 2.5.

Table 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

2.5 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 native 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).

2.5.1 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 [0.2% (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.

2.5.2 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 Ultrapure Milli-Q water. 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 Ultrapure Milli-Q water (3 x 20 s) and soaked in impregnation solution (20 min). After rinsing with

Ultrapure Milli-Q water (3 x 20 s), the gel was immersed in development solution until bands became visible. The gel was rinsed with Ultrapure Milli-Q water , treated with stop solution, and stored in a sealed plastic bag at 4°C until needed.

2.6 Protein fractionation using three phase partitioning (TPP)

TPP is an upstream purification method used to selectively concentrate proteins. Tertiary butanol is usually completely miscible with water, but upon the addition of 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 when 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 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 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 allowing it 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 (Dennison and Lovrien, 1997).

TPP has been used to purify the lysosomal proteinases cathepsins L (Pike and Dennison, 1989b), D (Jacobs *et al.*, 1989), S (Meinesz, 1996) and B (Meinesz, 1996). In this study TPP was used to purify bovine liver cathepsins B and D.

2.6.1 Procedure

t-Butanol was mixed into the aqueous solution to be fractionated to a final volume of 30% (v/v). The volume of t-butanol required was calculated form the equation:

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

where y = volume of t-butanol
x = volume of aqueous solution

t-Butanol crystallises below 25°C and was therefore warmed to 30°C prior to use. 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, 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.

2.7 Hydroxyapatite chromatography

In the purification of cathepsin S, Meinesz (1996) employed hydroxyapatite (HA) chromatography to separate cathepsin S from two contaminating bands that could not be removed by conventional ion-exchange or molecular exclusion chromatography. The resolving power of the technique is based on the differing retention mechanisms for acidic and basic proteins. These differing mechanisms of binding can allow the hydroxyapatite column to behave as a cation and an anion exchanger in a single run. The mechanism of hydroxyapatite chromatography has been described by Gorbunoff (1985).

Positive amino groups adsorb electrostatically to the negatively charged phosphate groups on hydroxyapatite :

hydroxyapatite- PO_4^-----H_3^+N-protein

Carboxylic acids groups are repelled by negative charges on the column. These moieties bind by complexation to calcium sites on the column:

hydroxyapatite Ca^{2+}----^-OOC-protein

hyroxyapatite -PO_4^----- Ca^{2+}----^-OOC-protein

Like in conventional ion-exchange chromatography, factors like the pI, charge distribution and the concentration of the protein of interest will determine the affinity with which a basic or acidic protein binds to a column.

There are two methods available to displace amino groups from the column. Firstly Debye-Huckle charge screening uses anions like F^-, Cl^-, ClO^-, SCN^- and phosphate to disrupts the amino phosphate interaction. Alternatively, calcium and magnesium ions which complex the column phosphates can be used to displace the amino-phosphate interactions:

$$HAPO_4^-\text{----}H_3^+N\text{-protein} + CaCl_2 \rightarrow HAPO_4^-\text{----}Ca^{2+} + 2\ Cl^- + H_3^+N\text{-protein}$$

Carboxylic acid groups are displaced from their hydroxyapatite-calcium sites by ions, like fluoride or phosphate, which form stronger complexes with calcium than do carboxyls:

$$HACa^{2+}\text{----}^-OOC\text{-protein} + NaF\ (PO_4^-) \rightarrow HACa^{2+}\text{----}F^-\ (PO_4^-) + Na^+ + {}^-OOC\text{-protein}$$

The formation constants for the calcium fluoride or calcium phosphate complexes are very much greater than the calcium carboxyl complexes, and hence the displacement of the carboxylic acids occurs at relatively low molarities of fluoride or phosphate.

The hydroxyapatite medium is in the form of blade-like crystals. As a result, the column has a weak binding capacity when compared to conventional bead media. Further, the brittle blade-like crystals tend to form fines. These drawbacks mean that hydroxyapatite chromatography is best employed downstream in a purification procedure. The versatility of the column, however, does tend to outweigh these disadvantages.

2.8 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).

2.8.1 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. The 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) as a preservative.

CHAPTER 3

ISOLATION OF THE LYSOSOMAL ENZYMES, CATHEPSIN B AND CATHEPSIN D

3.1 Introduction

Since de Duve's classical studies on lysosomes (de Duve, 1983; Bainton, 1981), advances in cell biology have shown that endocytosis and proteolysis along the endocytic pathway are complex and dynamic processes. As the envisioned pathway has become more complex, the roles assigned to the hydrolytic enzymes within the system has also undergone revision.

The lysosomal 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 reaborption 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). Cathepsin D [EC 3.4.23.5] is an aspartic protease that is also widely distributed throughout mammalian cells. Like cathepsin B, the main role of cathepsin D within the lysosome 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).

In order to undertake the studies described in Chapter 4, purification schemes that were rapid and cheap had to be developed for isolating these enzymes. The purification schemes used in this study were based on previous studies in this laboratory on the isolation of cathepsin B (Meinesz, 1996) and cathepsin D (Jacobs *et al.*, 1989) from bovine spleen. In this study the enzyme was isolated from bovine liver, and the differences in the purification protocols reflect the differences in protein composition between the two tissues.

3.2 Cathepsin B

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-endosomal 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 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 (MPR's). These receptors deliver the pro-enzyme to the endosomal system, where the acidic conditions trigger the dissociation of the enzymes from their MPR's (Hasilik, 1992). Pro-cathepsin B may also reach the endosomal 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 endosomal system, it is activated either by 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 endosomal system (Musil *et al.*, 1991).

3.2.1 Fluorometric assay for cathepsin B and cathepsin L activities

The fluorometric substrate CBz-R-R-AMC has proved to be an ideal substrate 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 at the P1 position, where it can be electrostatically anchored to a glutamic acid in the S1 position (Musil *et al.*, 1991). While cathepsin B can cleave the substrates CBz-R-AMC and CBz-F-R-AMC, these can be also 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 the CBz-R-R-AMC substrate, 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.

3.2.1.1 Reagents

Buffer/activator [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% NaN_3, 5 mM dithiothreitol, 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 -4°C. When required, this stock was diluted in dist.H_2O to a working concentration of 40 µM .

1 mM CBz-F-R-AMC substrate stock solution. CBz-F-R-AMC (1 mg) was dissolved in DMSO (1.5 ml), divided into 100 µl aliquots. When required, this stock was diluted in dist.H_2O to give a working concentration of 20 µM.

Diluent [0.1% Brij]. Brij (0.1 g) was dissolved in 95 ml of distilled water 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.

3.2.1.2 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.

3.2.2 Azocasein assay

An attempt was made to develop a protocol that allowed the co-purification of a related protease, cathepsin L, during the cathepsin B isolation. Although this attempt was unsuccessful, aspects of the co-purification protocol, and assays employed, are described because of their possible application in future studies.

Azocasein is a casein derivative in which the tyrosyl and histidyl side-chains have been coupled with diazotized sulfanilic acid or sulfanilamide in an alkali medium. Proteolytic cleavage of the azocasein generates peptides which are TCA-soluble. The azo-coupling confers an intense yellow colour to these peptides which can be quantified by their A_{334}. This assay can be made specific for cathepsin L by including 3 M urea and pepstatin into the assay. Whilst cathepsin L is stable in 3 M urea, these conditions should denature cathepsin B. Pepstatin was also included in the assay buffer to inhibit cathepsin D (Barrett and Kirschke, 1981).

3.2.2.1 Reagents

Buffer/activator [340 mM Na-acetate, 4 mM EDTA, 1 µg/ml pepstatin, 0.02% NaN_3, 8 mM DTT, pH 5.0]. Glacial acetic acid (9.72 ml), EDTA (0.744 g), NaN_3 (0.1 g) and pepstatin (0.5 mg) were dissolved dist.H_2O, adjusted to pH 5.0 with NaOH and made up to 500 ml. DTT (6.2 mg/5 ml) was added to the buffer just before use.

6% (m/v) Azocasein. Azocasein (3 g) was weighed into a glass beaker and dissolved in 50 ml dist.H_2O with gentle magnetic stirring at room temperature for about 1 h.

Azocasein/3M urea solution. Urea (54 g) was dissolved in 6% azocasein solution (50 ml) by magnetic stirring, and made up to 150 ml with dist.H_2O.

5% (m/v) TCA. TCA (25 g) was dissolved in 500 ml of dist.H_2O.

3.2.2.2 Procedure

Enzyme sample (200 µl) and buffer/activator (200 µl) were mixed and incubated at 37°C for 5 min to activate the proteases. Azocasein/urea (400 µl) was added to this solution and a sample (200 µl) of the mixture was immediately withdrawn, and mixed with 5% TCA in a polyethylene microfuge tube. This served as zero time control for the reaction. After 30 min, a further sample was withdrawn and mixed with 5% TCA (1 ml). The samples in the 1.5 ml microfuge tubes were centrifuged in a Sigma mini-centrifuge (12 000 x g, 5 min, RT) to precipitate undigested azocasein. The A_{334} of the supernatants were read in glass micro-cuvettes with blacked-out sides

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

In the protocol developed by Meinesz (1996), the TPP cut of 30-40 % resulted in a loss of yield of *ca.* 99% and a purification of only 4.2-fold. Whilst this cut allowed for the rapid isolation of electrophoretically pure cathepsin B, the amount of enzyme produced per isolation would have been insufficient for the purposes of the present study. Consequently it was necessary to reinvestigate the TPP fractionation step in order to determine an optimal cut.

3.2.3.1 Reagents

Homogenisation buffer [50 mM Na-acetate, 150 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, 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.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, 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.

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

3.2.3.2 Procedure

Bovine liver was allowed to thaw overnight (12 h) at 4°C to effect release of lysosomal hydrolases from their endocytic compartments. The liver was homogenised in homogenisation buffer at a ratio of 1:1 (liver mass to buffer) in a Waring blendor (max. speed, 3 min/100 g of tissue), and 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 2.6. An initial ammonium sulfate cut of 10% (m/v) was used, the precipitate being collected and dissolved in buffer A (50 ml). Empirically it was found that the 10% (m/v) ammonium sulfate cuts were heavily contaminated with cellular debris and this cut was therefore not analysed further. Further cuts with 5% (m/v) increments of ammonium sulfate were made to the solution. The precipitates at each step were collected and dissolved in buffer A (50 ml). These solutions were assayed for protein content by the Bradford assay (Section 2.2.1) and for protease activity (Sections 3.2.1 and 3.2.2).

3.2.3.3 Results

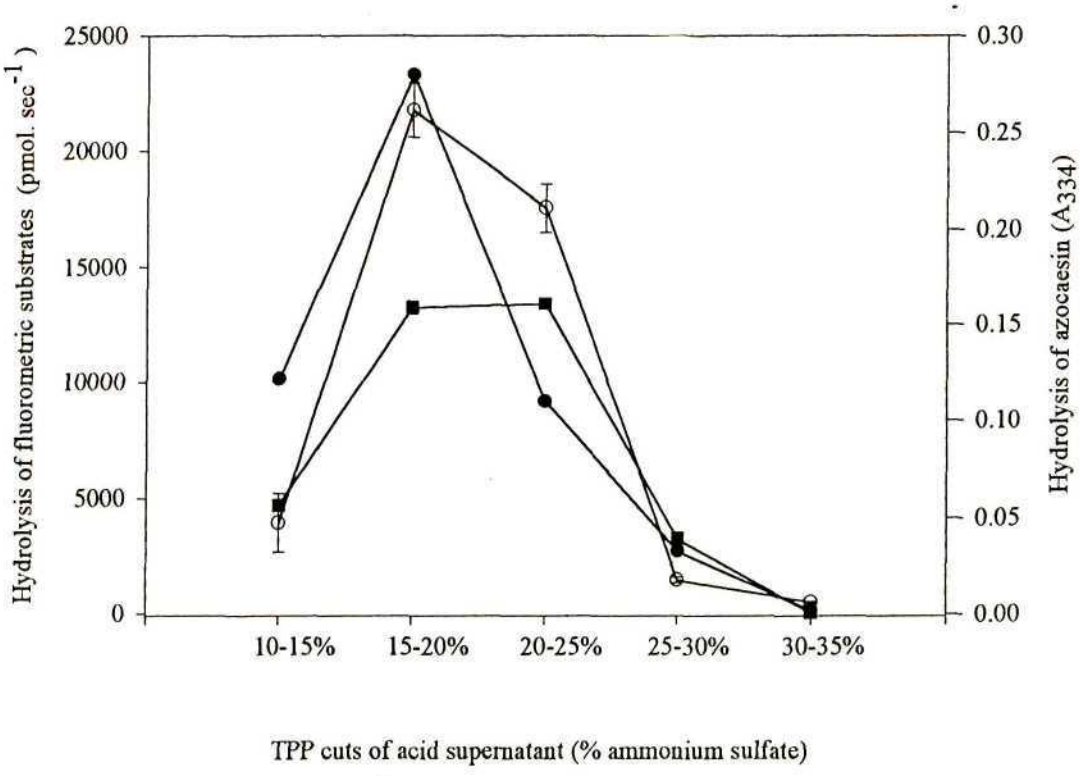

Figure 4. Optimal ammonium sulfate cuts for the isolation of cathepsin B.

As described in Section 3.2.3.2 an acid supernatant was fractionated using 5% ammonium sulfate cuts. The precipitate from each cut was assayed using CBz-R-R-AMC (○), CBz-F-R-AMC (■) and azocasein (●). The data represents mean ± s.d. (n=3).

3.2.3.4 Discussion

Judging by the hydrolysis of the substrate CBz-R-R-AMC, it appears that most of the cathepsin B precipitates in the 10-25% ammonium sulfate cut during TPP (Fig. 4). The 25-40% ammonium sulfate cut yields pure cathepsin B, but the yield obtained using this cut was significantly less than in the 10-25% cut (data not shown). When the substrate CBz-F-R-AMC was used, two distinct peaks of activity in the 15-20 % and 20-25% cuts, were obtained. The first peak optimum at 15-20% corresponds closely with the pattern of hydrolysis by cathepsin B of the substrate CBz-R-R-AMC. The second peak optimum probably falls in the 20-25% region and is responsible for the plateau shape of the CBz-F-R-AMC curve. This suggests that cathepsin L precipitates at higher ammonium sulfate concentrations (15-30%) than cathepsin B during TPP.

These results were confirmed using an azocasein assay, in the presence of 3 M urea. This assay revealed that maximum hydrolysis of the azocasein substrate occurred in the 15-20% ammonium sulfate cut, the same region where cathepsin B is optimally precipitated. This suggests that cathepsin B may be able to cleave azocasein even in the presence of 3 M urea. Whilst pure cathepsin B is sensitive to 3 M urea (Khan *et al.*, 1992), a membrane associated cathepsin B in fibroblasts and melanoma cells was shown to be insensitive to the urea (Mayer *et al.*, 1997). Whilst most membranes are expected to partition into the tertiary butanol phase during TPP, a possible explanation for the apparent insensitivity of cathepsin B to urea insensitivity could be the association of the enzyme with other cellular components present in a crude mixture.

3.2.4 Isolation of bovine cathepsin B.

Cathepsin B is ubiquitously distributed and has been isolated from a variety of tissue sources. The following schemes highlight some of the tissue sources, isolation procedures and yields used to obtain pure cathepsin B. A distinction is made between the total amount of enzyme recovered (mg/kg of starting material), and the total amount of activity recovered (yield), from a purification.

Scheme I (Barrett and Kirschke, 1981)

Step	Procedure
Starting material	Human liver
1	Homogenisation
2	Autolysis overnight
3	Acetone fractionation
4	Dialysis overnight
5	Chromatography on DEAE cellulose
6	Chromatography on Aminophenylmercuric acetate-Sepharose
Yield	60 mg/kg

Scheme II (Willenbrock and Brocklehurst, 1985a)

Step	Procedure
Starting material	Bovine spleen
1	Homogenisation
2	Acid precipitation overnight
3	Ammonium sulfate precipitation
4	Dialysis and concentration
5	Sephadex G-75 chromatography
6	Thiol addition and desalting
7	Thiopropyl-Sepharose 6B affinity chromatography
8	CM cellulose cation exchange chromatography
Yield	No yield recorded

Scheme III (Bradley and Whitaker, 1986)

Step	Procedure
Starting material	Bovine brain
1	Homogenisation
2	Acetone fractionation
3	Chromatography on CM-Sephadex
4	Gly-Phe-Phe-cysteamine-Affigel 10 affinity chromatography
5	Ultrogel AcA 54 molecular exclusion chromatography
Yield	0.64 mg/kg of tissue, 21.1 % yield

Scheme IV (Rich *et al.*, 1986)

Step	Procedure
Starting material	Human liver
1	Homogenisation
2	Freeze-thaw
3	Autolysis overnight
4	Acetone fractionation
5	Sepharose-Ahx-Gly-Phe-GlySc chromatography
Yield	17.33 mg/kg of tissue, 171% yield

Scheme V (Deval *et al.*, 1990)

Step	Procedure
Starting material	Bovine liver
1	Homogenisation
2	Freeze-thaw
3	Centrifugation
4	DEAE Zeta-Prep-Disk
5	Sephadex G-75 chromatography
6	Mono-S chromatography
7	Organomercurial-Sepharose chromatography
Yield	0.6 mg/kg of tissue, 3.9% yield

Scheme VI (Meinesz, 1996)

Step	Procedure
Starting material	Bovine spleen
1	Homogenisation
2	Acid precipitation
3	TPP
4	S-Sepharose cation exchange chromatography
5	Sephacryl S-100 chromatography
6	Hydroxyapatite chromatography
Yield	0.00689 mg/kg, 0.05% yield

Cathepsin B has been cloned and expressed in yeast. Two schemes (VII and VIII) describing the purification of the protease are described below:

Scheme VII (Illy *et al.*, 1997)

Step	Procedure
Starting material	Bovine liver
1	Cathepsin B expressing clone of *E. coli*
2	Transformation into *Pichia pastoris* GS 115
3	Induction of expression of recombinant enzyme
4	Harvest and concentration of culture supernatant
5	Dialysis
6	S-Sepharose or Sephacryl S-200 HR
Yield	20 mg/litre of culture medium

Scheme VIII (Björk *et al.*, 1994)

Step	Procedure
Starting material	Bovine liver
1	Cathepsin B expressing clone of *E. coli*
2	Transformation into yeast (species not given)
3	Induction of expression of recombinant enzyme
4	Harvest and concentration of culture supernatant
5	Acetone precipitation
6	DEAE Sepharose
7	Thiopropyl Sepharose
Yield	2.2 mg/litre of culture medium

Meinesz (1996) developed a cost-effective and rapid isolation procedure for cathepsin B from bovine spleen (Scheme VI). Electrophoretically pure enzyme could be obtained within 48 h, using readily available chromatographic media. However, the yield of enzyme obtained was very low, especially compared to the protocol of Rich *et al.* (1986) (Scheme IV) which uses Sepharose-Ahx-Gly-Phe-GlySc affinity chromatography. The Sepharose-Ahx-Gly-Phe-GlySc affinity chromatography gel is no longer commercially available, however, and its synthesis is expensive and technically complex. The other procedures described may also give higher yields of enzyme, but at the cost of time (Schemes I-III, V), expense (Scheme I, III, IV and V) or technical complexity (Schemes VII and VIII). Consequently in this study an attempt was made to modify the procedure of Meinesz (1996), with its advantages of convenience, low cost and simplicity, to give improved yields of enzyme .

3.2.4.1 Reagents

Homogenisation buffer [50 mM Na-acetate, 150 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, 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.

Buffer A [20 mM Na-acetate, 1 mM Na_2EDTA, 0.02% NaN_3, 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 B [100 mM Na-acetate, 500 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3, pH 5.5]. Glacial acetic acid (11.44 ml), NaCl (58.44 g), Na_2EDTA (0.74 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of dist.H_2O, adjusted to pH 5.5 with NaOH and made up to 2 litres.

Buffer C1 [1 mM Na-phosphate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 6.8]. $NaH_2PO_4.H_2O$ (0.159 g), Na_2EDTA (0.37 g) and NaN_3 (0.2 g) were dissolved in 950 ml of dist.H_2O, adjusted to pH 6.8 with NaOH and made up to 1 litre.

Buffer C2 [10 mM Na-phosphate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 6.8]. $NaH_2PO_4.H_2O$ (0.793 g), Na_2EDTA (0.185 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 6.8 with NaOH and made up to 500 ml.

Buffer C3 [300 mM Na-phosphate, 1 mM Na_2EDTA, 0.02% NaN_3, pH 6.8]. $NaH_2PO_4.H2O$ (23.80 g), Na_2EDTA (0.185 g) and NaN_3 (0.1 g) were dissolved in 450 ml of dist.H_2O, adjusted to pH 6.8 with NaOH and made up to 500 ml.

"Buffer" D [1 mM $MgCl_2$, 1 mM Na_2EDTA, 0.02% NaN_3]. $MgCl_2.6H_2O$ (0.218 g), Na_2EDTA (0.185 g) and NaN_3 (0.1 g) were made up to 500 ml with dist.H_2O.

"Buffer E" [1 M NaCl, 1 mM Na_2EDTA, 0.02% NaN_3]. NaCl (29.22 g), Na_2EDTA (0.185 g) and NaN_3 (0.1 g) were made up to 500 ml with dist.H_2O.

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.

Biogel P-60. The Biogel P-60 gel was made by hydrating the xerogel with buffer A (11 ml per gram of gel). The column bed was initially regenerated using 5 column volumes of buffer B. In between purification procedures the column was re-equilibrated with 2 column volumes of buffer B. The gel was calibrated using the molecular weight standards, blue dextran (>2 000 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), myoglobin (18.8 kDa) and cytochrome C (12.38 kDa).

Hydroxyapatite. The hydroxyapatite resin was prepared according to the manufacturer's instructions. The hydroxyapatite gel was mixed with 2 volumes of buffer C1 and left to settle into two distinct phases. After 10 min, the upper layer, containing any fines that may have been generated during handling, was removed and replaced by 2 more volumes of buffer C1. This procedure was repeated. These wash steps are necessary because the brittle nature of the hydroxyapatite crystals often results in the generation of fines that can block columns. The remaining slurry was packed into a glass column under gravity. The column was regenerated and equilibrated with five column volumes of buffer C1.

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.

3.2.4.2 Procedure

Bovine liver was homogenised and acid precipitated as described in Section 3.2.3.2. The acid supernatant was subjected to TPP (Section 2.6.1) using an ammonium sulfate cut of 10-25%. The TPP precipitate was dissolved in buffer A at 1/10 the volume of the acid precipitate. This sample was loaded directly onto an S-Sepharose column (2.5 x 17.5 cm = 85.9 ml), equilibrated with buffer A, at 10 cm h^{-1}. Cathepsin B was eluted with a gradient of 70-200 mM NaCl in buffer A. Active fractions, which eluted between 90-150 mM NaCl, were pooled and dialysed against sucrose to a volume of approximately 1-5% of the volume of the molecular exclusion gel. These fractions were loaded onto a Biogel P-60 column (2.5 x 91.5 cm = 444.9 ml) and run at 5 cm h^{-1}. Active fractions were analysed by SDS-PAGE (Section 2.4) and silver staining (Section 2.5) to determine their purity.

If these fractions were not pure they were pooled and dialysed against several changes of buffer C1. The pooled fraction was applied to a hydroxyapatite column (1.5 X 7.7 cm = 13.6 ml), equilibrated with buffer C1, at 5 cm h^{-1}. Cathepsin B was purified using a scheme devised by Gorbunoff (1985). This scheme allows for the separation of basic, neutral and acidic proteins using the principles described in Section 2.7. Basic proteins were eluted by washing the column with two volumes of "buffer" D. Neutral proteins were then eluted by washing the column with two volumes of "buffer" E. Cathepsin B, which elutes in the acid fraction, was eluted using a gradient of 10-300 mM NaH_2PO_4 (buffer C2 and buffer C3). Purity of the active fractions was assessed by SDS-PAGE (Section 2.4) and silver staining (Section 2.5).

3.2.4.3 Results and discussion

Table 2. The purification of cathepsin B from bovine liver

	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (fold)	Yield (%)
Homogenate	2 934	354.42	0.120	1	100
Acid Ppt.	2 435	312.50	0.128	1.063	88
TPP (10-25%)	183	180	0.98	8.167	50.78
S-Sepharose	15.32	30	1.957	16.308	8.46
Biogel P-60	1.07	15.82	14.75	122.917	4.46

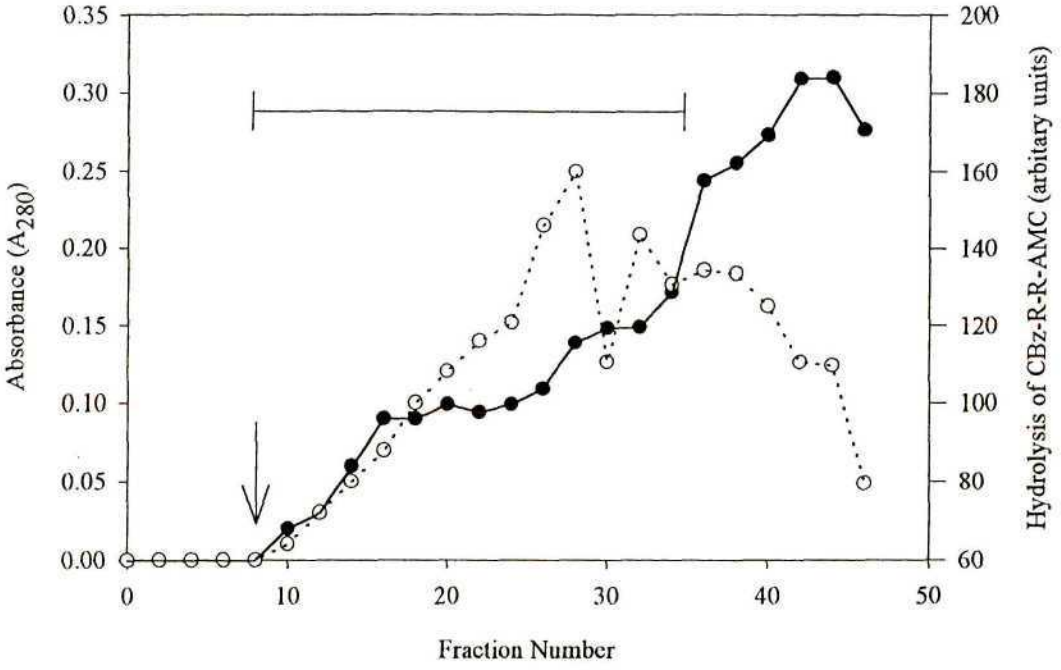

Figure 5. Chromatography of the pH 4.2 TPP fraction of cathepsin B on S-Sepharose.

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 70-200 mM NaCl gradient was applied at ↓; flow rate 10 cm h^{-1}; fractions, 8.3 ml. (-•-) A_{280}, (-o-) cathepsin B activity, (I-I) pooled fraction.

The modifications introduced into the protocol of Meinesz (1996) resulted in an improved cathepsin B yield of 4.46% compared to the 0.05% obtained with the original protocol. Whilst the yield obtained by this method is significantly less than that obtained by Rich *et al.* (1986) and Barrett and Kirschke (1981), its relative convenience, cost-effectiveness and simplicity make it an attractive alternative to these methods. This method proved to be better than the methods described by Deval *et al.* (1990) and Bradley and Whitaker (1986) in terms of the amount of purified enzyme obtained per kg of starting material and the simplicity of procedure.

The first modification made was at the TPP level. An ammonium sulfate cut of 10-25% (Section 3.2.3) was used during TPP, rather than the 30-40% and 25-40% ammonium sulfate cuts described by Meinesz (1996). This change results in a different profile of proteins being included in the TPP cut, necessitating changes in the downstream purification procedures. The general applicability of this method was emphasised by the fact that cathepsin B could be purified from a 25-40% cut with a yield of 0.48% (data not shown). Thus pure enzyme could be obtained using a 10-25% and a 25-40% ammonium sulfate TPP

cut. The 25-40% cut was not routinely used however, because of the effectiveness of the 10-25% cut.

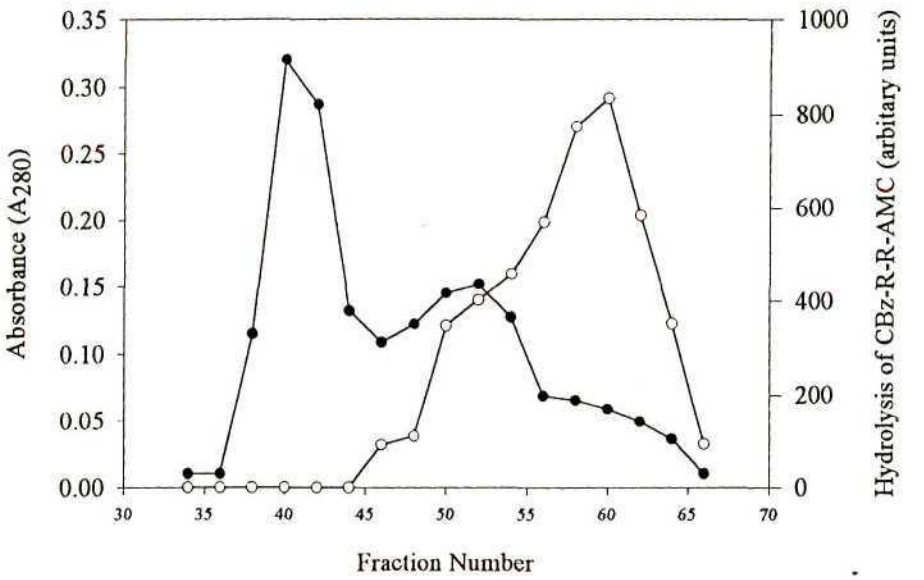

Figure 6. Chromatography of pH 5.0 S-Sepharose fraction on Biogel P-60.

Column, 2.5 x 91.5 cm (445 ml bed volume); buffer, 100 mM Na-acetate, pH 5.5, containing 500 mM NaCl, 1 mM Na_2EDTA, 0.02 % NaN_3; flow rate 5 cm h^{-1}; and fractions 5 ml. (-•-) A_{280}, (-○-) cathepsin B activity. Fraction purity was assessed by SDS-PAGE and pooled accordingly.

This success of this purification procedure hinges largely on the performance of the S-Sepharose cation exchanger. As described by the increase in specific activity at this stage (Table 2), the S-Sepharose cation exchanger is responsible for removing a large proportion of the contaminants that could co-elute with cathepsin B in the molecular exclusion step following cation exchange chromatography. It was found that the profile of proteins coming off the cation exchanger was affected by the tissue batch and amount of tissue used, as well as slight changes in the homogenisation and acid precipitation steps. An advantage of methods that use affinity chromatography (see Schemes I-VI, Section 3.2.4) is that they are not as susceptible to changes in upstream methods. In order to counter this effect, two further changes were made to the original procedure. Cathepsin B was eluted off the S-Sepharose cation exchanger with a shallower salt gradient of 70-200 mM NaCl in buffer A (Fig. 5). This gradient allowed for greater selectivity in choosing fractions used for subsequent purification steps. An additional downstream step, using hydroxyapatite chromatography to purify contaminated fractions, was also introduced.

An attempt was made to co-purify cathepsin B and cathepsin L. It was envisaged that a 10-30% ammonium sulfate cut during TPP would be sufficient to harvest both enzymes (Section 3.2.3). Following TPP, the precipitate was loaded onto the S-Sepharose cation exchanger and cathepsin B eluted as described above. Cathepsin L, which binds to the column with greater affinity, was eluted with a gradient of 200-700 mM NaCl in buffer A (data not shown). However, the altered protein profile produced by the new 10-30% ammonium sulfate cut did not allow efficient purification of either protease.

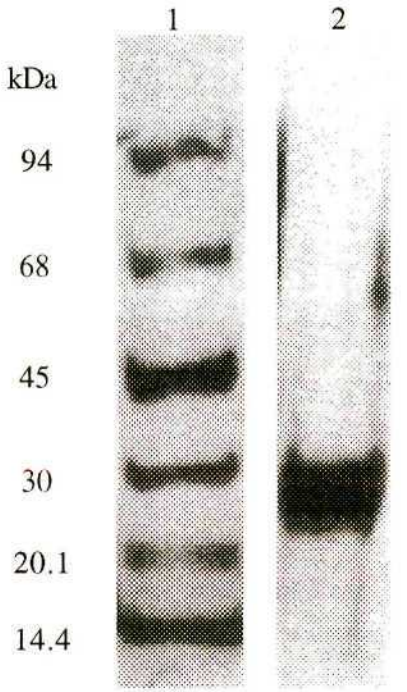

Figure 7. Tris-tricine SDS-PAGE of cathepsin B isolated from bovine liver.

Pure cathepsin B fractions from the P-60 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 kDa) are indicated in lane 1.

A Biogel P-60 molecular exclusion gel was used to purify cathepsin B from fractions pooled at the S-Sepharose step (Fig. 6). Biogel P-60 was used in place of the Sephacryl S-100 gel used by Meinesz (1996) because of the presence of a contaminating protein of approximately 70 kDa that could not be satisfactorily resolved using the Sephacryl gel. This contaminating band was precipitated in the 10-25% ammonium sulfate TPP cut, but did not appear in the 25-40% TPP cut (data not shown). Although a single chain enzyme is indicated in Fig. 7, both single and two-chain forms of cathepsin B were obtained during the purification.

If the cathepsin B fractions appearing after molecular exclusion were still contaminated they were chromatographed on hydroxyapatite as described above. This additional procedure poses the question as to why hydroxyapatite was not used directly after S-Sepharose cation exchange chromatography. The answer is that the brittle nature of the hydroxyapatite gel and its low binding capacity made it most suitable only for a final step.

3.2.5 Active site titration of cathepsin B using E-64

Active site titration of cathepsin B was undertaken in order 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 that was 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 abolish enzyme activity (Barrett and Kirschke, 1981).

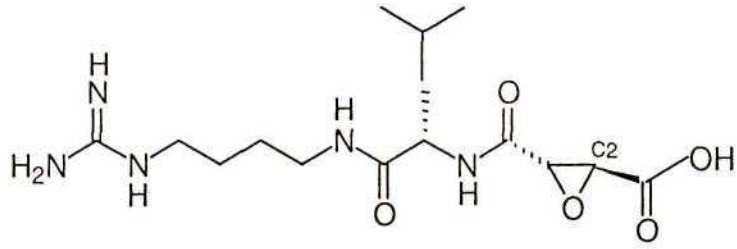

Figure 8. The structure of trans-epoxysuccinyl-leucylamido(4-guanidino) butane (E-64).

The C2 position is the site for the formation of the irreversible thioether bond between E-64 and cathepsin B.

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 in this active site titration were as described by Barrett and Kirschke (1981).

Active site titration was routinely used to determine the absolute concentration of enzyme used in the assays described in Chapter 4, as well as to monitor changes in enzyme activity during enzyme storage.

3.2.5.1 Reagents

Buffer/activator [0.1 M Na-phosphate, 4 mM Na_2EDTA, 0.02% NaN_3, 5 mM dithiothreitol, 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 DMSO (5 ml), divided into 100 µl aliquots and stored at -4°C. When required, this stock was diluted in dist.H_2O to a working concentration of 40 µM .

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).

3.2.5.2 Procedure

The active site titration of cathepsin B was adapted from Barrett and Kirschke (1981). Enzyme sample (25 µl), buffer/activator (50 µl) 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 3.2.1) and a graph of activity versus E-64 concentration constructed. The absolute concentration of active enzyme (E_0) was determined from the intersection of the graph with the E-64 concentration axis.

3.2.5.3 Results

Using active site titration, the absolute concentration of isolated enzyme was calculated to be 1.58 µM. This represents 30% of the purified sample.

3.3 Cathepsin D

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 endosomal system by the mannose 6 phosphate receptor system (MPR). 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 (Rijnbout *et al.*, 1991a; 1991b) and mouse fibroblasts (McIntyre and Erickson, 1991;1993). This discrepancy in the results obtained suggests that MPR-independent transport systems may be cell specific.

Upon delivery to the endosomal system, procathepsin D is proteolytically processed to a mature enzyme. In rat, mouse and hamster tissue the enzyme is processed to a single chain enzyme, 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)

The 44 kDa single chain enzyme can, depending on the species concerned, be processed into a 2-chain form. The light (15 kDa) and heavy chains (30 kDa) 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).

3.3.1 Cathepsin D assay

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 at using 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.

3.3.1.1 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.

3.3.1.2 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 polyethylene microfuge 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 reaction mixture supernatant was read against the blank supernatant in a 1 ml quartz cuvette at 280 nm. In this study, 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.

3.3.2 TPP optimisation for cathepsin D purification from bovine liver.

Previous cathepsin D isolations undertaken in this laboratory from bovine, porcine and human spleens have used an optimal TPP cut of 20-35% ammonium sulfate (Fortgens, 1996). However, an optimal TPP cut has not been described for bovine liver cathepsin D. In order to determine this cut, a TPP optimisation was undertaken along similar lines to that described for cathepsin B (Section 3.2.3).

3.3.2.1 Reagents

Loading buffer [50 mM Na-acetate, 200 mM NaCl, 0.02% NaN_3, pH 3.5]. Glacial 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 3.2.3.1.

3.3.2.2 Procedure

Bovine livers were allowed to thaw overnight (12 h) at 4°C to effect the release of cathepsin D from the endosomal-lysosomal system. These livers were homogenised in cold dist.H_2O at a ratio of 1:2 (liver mass to dist.H_2O) using a Waring blendor (max. speed, 3 min/100 g liver tissue). Undissolved protein was removed by centrifugation (10 000 x g, 30 min, 4°C) and the supernatant poured off. The pH of the supernatant was adjusted to pH 3.2 with HCl with constant stirring. Precipitated protein was removed by centrifugation (10 000 x g, 30 min, 4°C) and TPP effected on the supernatant (Section 2.6) using a 10% ammonium sulfate cut. Empirically it was found that the 10% ammonium sulfate precipitate had a low specific activity and was discarded. Further ammonium sulfate cuts in 10% increments were effected on the supernatant. The precipitates from each cut were assayed using the cathepsin D assay (Section 3.3.1).

3.3.2.3 Results and Discussion

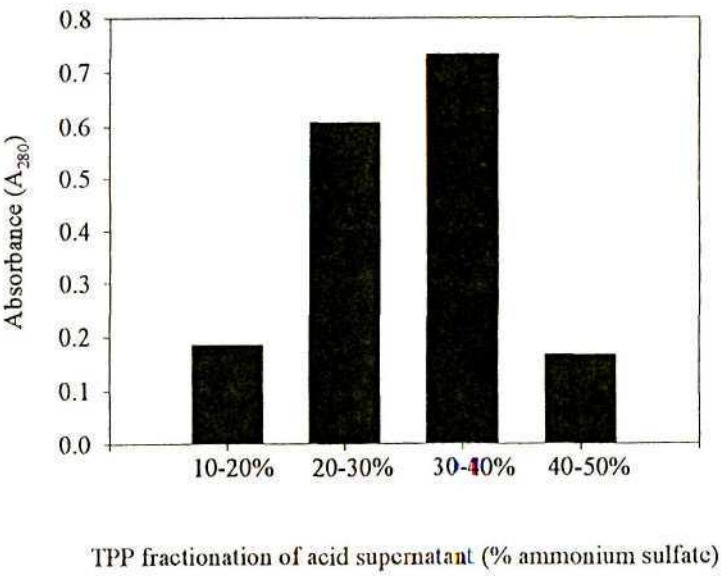

Figure 9. Optimal ammonium sulfate cuts for the isolation of cathepsin D.

As described in Section 3.3.2.2 an acid supernatant was fractionated using 10% ammonium sulfate cuts. The precipitate from each cut was assayed using haemoglobin.

The optimal TPP cut for the isolation of cathepsin D from bovine liver was between 20-35% ammonium sulfate (Fig. 9).

3.3.3 Purification of cathepsin D

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 2.6). An important property of TPP, that is especially relevant in this isolation, is the denaturation of many oligomeric proteins by the process. TPP causes the denaturation of haemoglobin, which as can be expected (Section 3.3.1), can be a major contaminant in cathepsin D isolations.

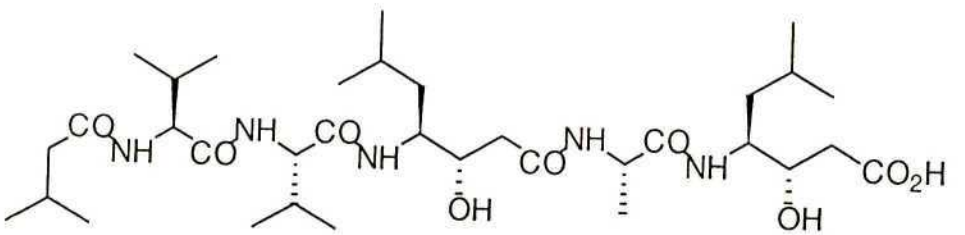

Figure 10. The structure of Isovaleryl-Val-Val-Sta-Ala-Sta (pepstatin A)

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 is a P1-P1' dipeptide isostere of Leu-Gly in which there is no P1' side-chain substituent (Fig. 10). 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.

3.3.3.1 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 3.2.3.1.

Pepstatin-diaminohexane-Sepharose. Preparation of this gel is described in Section 2.8.

3.3.3.2 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 2.6) using an ammonium sulfate cut of 20-35% (Section 3.3.2). The TPP precipitate was dissolved in loading buffer (Section 3.3.3.1) 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-diaminohexane-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 2.4) and silver staining (Section 2.5), and the fractions pooled accordingly.

3.3.3.3 Results and Discussion

Table 3. 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.	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

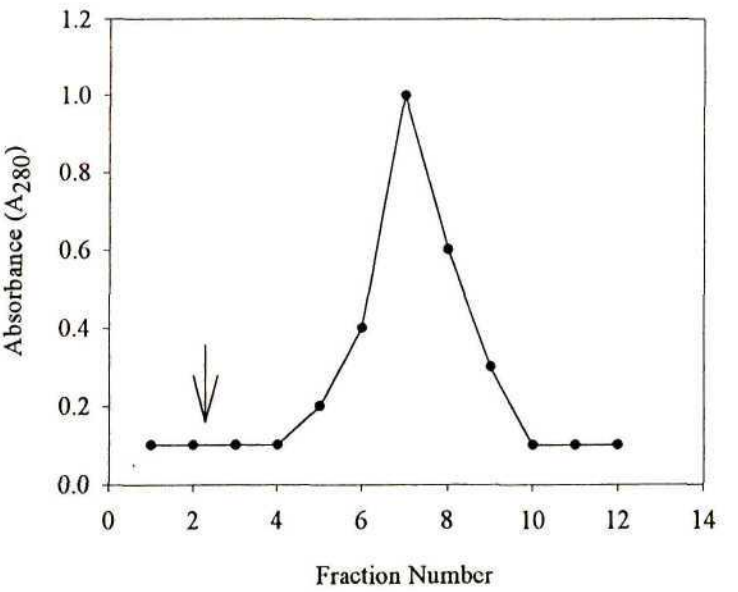

Figure 11. The elution of cathepsin D from pepstatin-Sepharose.

Pepstatin-Sepharose (Section 2.8) was equilibrated with loading buffer, and bound cathepsin D was eluted with elution buffer, all at 5 cm h^{-1}. The elution buffer was applied at (↓).

As expected, cathepsin D was isolated in the single (45 kDa) and two-chain (30 kDa and 15 kDa) forms (Fig. 12). The yield of purified cathepsin D (Table 3) was significantly less than that obtained by Jacobs *et al.*, (1989) . This is possibly due to the small size of the pepstatin affinity column employed in this study.

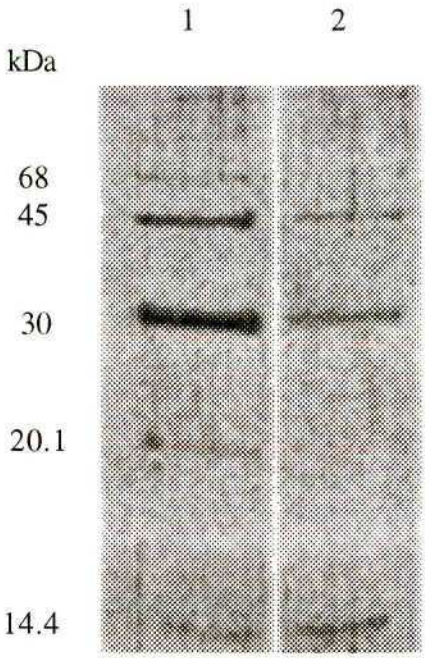

Figure 12. Tris-tricine SDS-PAGE of cathepsin D isolated from bovine liver.

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 kDa).

In order to complete the experiments described in Chapter 4 electrophoretically pure cathepsin B and cathepsin D were needed. An achievement of the studies described in this chapter, is that both enzymes could be isolated relatively rapidly and cost-effectively. The modifications introduced in the protocol described by Meinesz (1996) resulted in an improved yield of 4.46% when compared to the 0.05% yield of the original procedure. Significantly, the modifications introduced into the procedure involved changes that did not affect the cost or time-effectiveness of the original procedure. Cathepsin D was successfully isolated using an established procedure (Fortgens, 1996) although the yields obtained were lower. The amount of enzyme harvested from the purification was, however, sufficient for the studies undertaken in Chapter 4.

CHAPTER 4

THE REDUCTIVE ACTIVATION OF CATHEPSIN B

4.1 Introduction

Mammalian organisms have evolved a multi-tiered response to low oxygen levels, or hypoxia (Fig. 13). At the cellular level hypoxia induces a dramatic response in cells because aerobic metabolism is effectively inhibited. Cells switch over from oxidative phosphorylation to glycolysis as a means of generating ATP. Specific glycolytic isoenzymes like lactate dehydrogenase (LDH)-A, phosphofructokinase (PFK)-L, PFK-C, aldolase (ALD)-A, ALD-C, and the glucose transporters GLUT-1 and GLUT-2, are upregulated under conditions of low oxygen tension (Ebert *et al.*, 1996).

Oxygen tension is sensed by a haem-dependent NAD(P)H-oxidase, which under normal conditions binds free oxygen and converts it into hydrogen peroxide. The hydrogen peroxide is converted into hydroxyl and hydroxide radicals via the iron-mediated Fenton reaction. These reactive oxygen species (ROS) act as chemical messengers that suppress the expression of genes induced by hypoxia. Low oxygen tension, or the presence of certain transition metals (Co^{2+} and Ni^{2+}) which compete for the metal binding site on the oxygen sensor, disrupt the production of the ROS chemical messengers. This signal is relayed to the transcription factor hypoxic inducible factor-1 (HIF-1) which mediates the increase in transcription of the glycolytic enzymes described above. HIF-1 is a heterodimer that belongs to the PAS family of transcription factors. Whilst HIF-1α is a novel protein, HIF-1β had been previously identified as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 regulation may be regulated by protein stability, phosphorylation, redox modifications, or the retardation of complex formation with hsp90 (Huang *et al.*, 1997). HIF-1 is also responsible for the induction of transcription of the vascular endothelial growth factor (VEGF). VEGF stimulates the growth of blood vessels toward hypoxic tissue, decreasing the diffusion distance for oxygen entry into hypoxic cells.

At a systemic level, mammals respond to hypoxia by hyperventilation which increases arterial oxygen tension. This response is mediated by Type-1 cells of the carotid body. Low oxygen levels trigger the release of dopamine, whose production is upregulated in these cells. The rate limiting enzyme in dopamine biosynthesis is tyrosine hydroxylase, whose transcriptional upregulation is simulated by HIF-1 and the c-Fos-JunB AP-1 heterodimer (Norris and Millhorn, 1995).

A further systemic reaction to hypoxia is the transcriptional upregulation of the erythropoietin (Epo) gene. Epo is a 30.4 kDa glycoprotein hormone produced in the kidney and liver and is

upregulated between 50-100 times in response to hypoxia. Epo enters the plasma and binds to specific receptors on erythroid progenitor cells and precursor cells. These cells are stimulated to proliferate and differentiate into erythrocytes, increasing erythrocyte mass in the blood (Huang *et al.*, 1997).

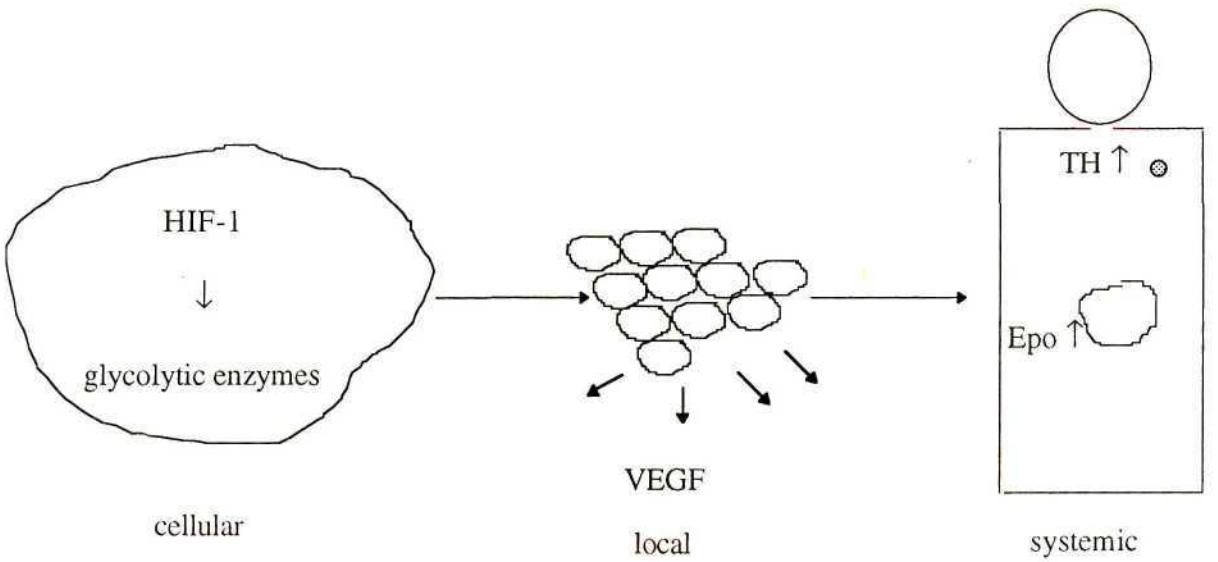

Figure 13. Cellular, local and systemic responses to hypoxia are mediated by HIF-1.

At a cellular level hypoxia activates HIF-1, which induces an increase in the transcription of specific glycolytic enzymes. HIF-1 activation also triggers the production of VEGF which stimulates the growth of blood vessels toward hypoxic regions. At a systemic level HIF-1 activation increases the delivery of oxygen to tissues by upregulating erythropoietin (Epo) and tyrosine hydroxylase (TH) production. The effects of these factors are discussed in the text.

Thus mammals have developed a hierarchy of systems to ensure that cells receive oxygen. Most oxygen delivered to cells is tetravalently reduced, by oxidative phosphorylation, to water. However, oxygen can also accept single electron transfers to produce the superoxide radical ($\cdot O_2^-$). Reduction of $\cdot O_2^-$ by superoxide dismutase (SOD) produces H_2O_2, which in turn is a substrate for catalases and peroxidases. H_2O_2 can combine with $\cdot O_2^-$ to produce the hydroxyl radical ($\cdot OH$). The hydroxyl radical is an extremely destructive species and can react with virtually any molecule within the cell. The $\cdot OH$ radical and other radical species can inactivate enzymes, break DNA, and initiate chain reactions that peroxidize lipids. Thus oxygen in high concentrations is toxic, with its toxicity being mediated through the production of ROS (Allen, 1991).

Despite their toxicity and danger to molecules in a cell, ROS are used as chemical messengers for oxygen sensing, and play a role in tissue differentiation. It has been shown that as cells become more differentiated, a process associated with a loss of mitotic activity, they become more oxidising. In contrast, tumour cells and cells with a high regenerative capacity like liver cells, maintain a more highly reducing cytosolic environment (Allen, 1991).

Thus, the maintenance of oxygen homeostasis has profound effects on metabolism and cell survival. Oxygen is necessary for oxidative phosphorylation where energy gains are greater than in glycolysis. ROS which are generated by the reduction of oxygen serve as important chemical messengers for oxygen homeostasis and differentiation. However, these ROS have the capacity to damage a large number of cellular components, and possibly kill cells. In order to protect against ROS, cells have developed several enzymatic and non-enzymatic mechanisms to counter their deleterious effects. The susceptibility of the enzymatic mechanisms to inactivation, suggests that non-protein oxidants are essential for dealing with ROS (Allen, 1991).

In lipids α-tocopherol and β-carotene are responsible for limiting lipid peroxidation by terminating chain reactions in membrane lipids. Hydrophilic antioxidants like GSH and ascorbate remove ROS species from the cytoplasm by reacting directly with them. Reduced glutathione (L-γ-glutamyl-L-cysteinylglycine), or GSH, is widely distributed throughout nature and is present in cells at a relatively high concentration, between 0.1-10 mM (Meister, 1995). Apart from being an antioxidant, GSH is the principal redox buffer within the cytoplasm and is responsible for maintaining a reducing cytosolic environment. Maintaining this redox environment is important for cellular polarity, compartmentalisation of ionic charges within the cell, and for protein function and charge (Allen, 1991). Further, GSH also functions as a storage and transport form of cysteine (Meister, 1995).

ROS and disulfides are reduced by GSH, which becomes oxidised to its disulfide form, GSSG. GSSG reductase, which utilises NADPH as a co-factor, reduces GSSG back to GSH. The enzyme GSSG-reductase catalyses an equilibrium that greatly favours the formation of GSH, which is therefore present at concentrations 100-400 times that of GSSG (Gilbert, 1995). NADPH, the co-factor for GSSG-reductase, is generated by oxidative phosphorylation, and ultimately provides the reducing power within the cell. Thus, the reducing power within a cell paradoxically depends on the delivery of oxygen to that cell (Meister, 1995).

This relationship between cellular redox status and its dependence on oxidative phosphorylation has received attention because of its application in ischaemia and secondary metastatic dissemination. Solid tumours often have microenvironments that are hypoxic, deprived of nutrients and of low pH. These environments select for apoptotically resistant

and aggressive phenotypes (Graeber *et al.*, 1996). Because these microenvironments do not receive an adequate oxygen supply, they (paradoxically) are subjected to oxidative stress, and tend to upregulate GSH production. GSH can detoxify many chemotherapeutic drugs (Cook and Mitchell, 1995). Thus the combination of the tumour microenvironment and chemotherapeutic drugs selects for those tumour cells that are extremely aggressive, and resistant to chemical therapy.

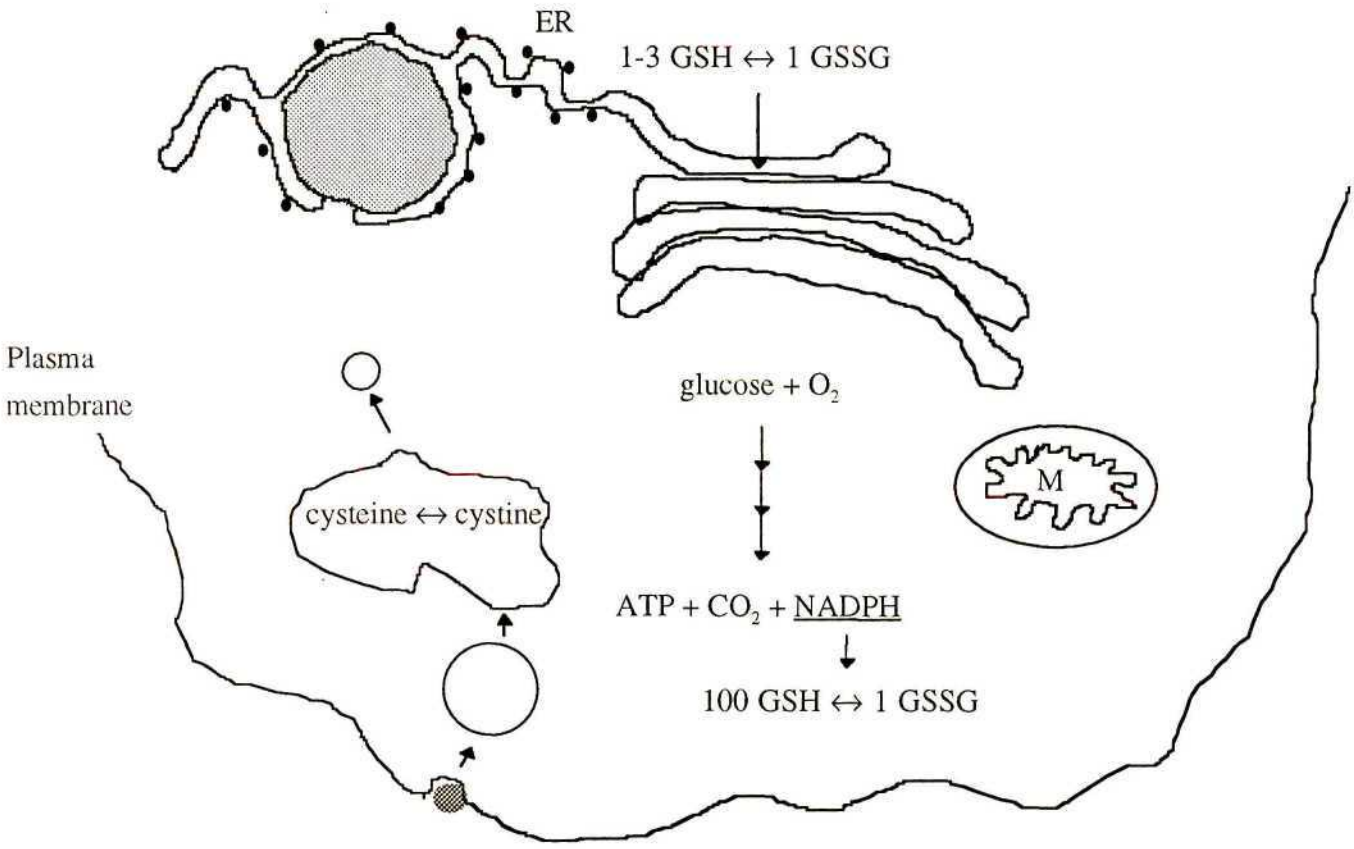

Figure 14. The redox environments within the cell.

The highly reducing environment within the cytoplasm is generated by GSH. The GSH:GSSG ratio is maintained by GSSG reductase which uses NADPH as co-factor. NADPH is produced by glycolysis and the Kreb's cycle, and thus all the cellular redox environments are modulated by oxygen delivery to the cell. The endosomal system has pumps that transport cysteine into the system to generate what is believed to be a reducing environment. The ER redox environment is kept relatively oxidising by GSSG transporters. This redox environment together with molecular chaperonins allows proteins to fold into their correct conformations. The mitochondrial (M) redox system uses non-thiol redox systems and was not examined during this study.

The ER possesses a GSH:GSSG redox system (Fig. 14). In contrast to the cytoplasmic redox environment, the redox environment within the ER is relatively oxidising. The ER

redox environment is maintained at a GSH:GSSG ratio of 1-3:1. This ratio has been shown to be optimal for protein folding, and for oxidoreductases like protein disulfide isomerase (PDI). This relatively oxidising redox equilibrium within the ER is maintained by GSSG and cystine transporters that transport the disulfides into the ER at a rate 10 times faster than for GSH and cysteine respectively (Hwang *et al.*, 1992).

The relatively oxidising environment of the ER thus favours protein disulfide formation. The chemistry of disulfide formation involves the transfer of 2 electrons from a thiol to an acceptor, resulting in the formation of a mixed disulfide. This bond is then attacked by a cysteine residue, resulting in the formation of an intrachain disulfide bond. The ER redox potential, as well as the microenvironment surrounding the cysteine residues, has thermodynamic and kinetic effects on the formation and interchange of the disulfide (Huppa and Ploegh, 1998). To ensure that the redox potential within the ER favours disulfide formation, it appears that the ER has proteins dedicated to maintaining the lumenal redox potential. Ero1p is a membrane associated glycoprotein that is ubquitously expressed in eukaryotic cells. Ero1p is probably part of a redox cascade responsible for maintaining an oxidising ER redox potential (Frand and Kaiser, 1998; Pollard *et al.*, 1998).

As described above, the ER maintains a relatively oxidising environment to facilitate the correct folding of a nascent polypeptide. The folding apparatus within the ER also includes chaperonins like Bip/Kar2p which functions like a molecular ratchet in importing proteins into the ER. Calnexin and calreticulin are Ca^{2+} dependent chaperones that bind unfolded substrate in a lectin-type fashion. Oxidoreductases like PDI, and isomerases like protein prolyl isomerase (PPI) also ensure that a nascent polypeptide assumes its stable and correct conformation (Huppa and Ploegh, 1998). Thus, the refolding apparatus within the ER appears to be very sophisticated and depends on both the redox environment and ER-resident enzymes to ensure that a polypeptide folds into its correct conformation.

The endosome-lysosome system on the other hand, serves to reverse this process. The redox environment within the late endosome-lysosome system must therefore favour the breaking of disulfide bridges, and support the (mainly cysteine) endoproteases involved in degrading endosomal substrates. In order to fulfil these requirements, the redox environment within the late endosome-lysosome system is expected to be considerably more reducing than that of the ER. Pisoni *et al.* (1990) have described a transporter which transports cysteine into the "lysosome". This transporter is believed to be responsible for maintaining a reducing endosomal environment. Lloyd (1992) has suggested that the stoichiometry of disulfide exchange would not necessitate a lysosomal transporter. He asserts that the cysteine transporter described by Pisoni *et al.* (1990), may constitute merely an anapleurotic pathway that replenishes cysteine lost by auto-oxidation However, if disulfide bond formation and thiol interchange are modulated by the redox environment (Huppa and Ploegh, 1998), a more

reducing environment would favour the breaking of disulfides and concomitant formation of thiols. This could be explained by the Nernst equation:

$$E = E_0 + \frac{2.3RT}{n\mathcal{F}} \log \frac{[\text{oxidised form}]}{[\text{reduced form}]}$$

where E is the reduction potential, E_0 is the standard reduction potential at pH 7.0, 25°C and at a gas pressure of 1 atm, [oxidised form] refers to the concentration of oxidising agent and [reduced form] refers to the concentration of reducing agent. As the concentration of reducing agent increases relative to oxidising agent, the environment would be expected to become more reducing, favouring disulfide breakage.

A point that is often overlooked about redox potentials is their dependence on pH. Under standard conditions (pH 7.00, 25°C, 1 atm), for every unit decrease in pH, the reduction potential for a reaction becomes more oxidising by a factor of 0.059 V (Segal, 1976). The following calculation highlights the effect that pH would have on the actual ratios of a redox buffer under standard conditions. Within the ER, the GSH:GSSG ratio is maintained at 3:1 to ensure that disulfide bond formation is favoured. The standard reduction potential for such a ratio can be calculated using the Nernst equation:

$$E = E_0 + \frac{2.3RT}{n\mathcal{F}} \log \frac{[\text{oxidised form}]}{[\text{reduced form}]}$$

$$\mathrm{E} = -0.23\mathrm{V} + \frac{0.059}{2} \log \frac{1}{3}$$

$$= \text{-0.244 V}$$

This potential can be expressed in terms of the cysteine:cystine the 'lysosomal' redox buffer (Pisoni and Thoene, 1991):

$$E = E_0 + \frac{2.3RT}{n\mathcal{F}} \log \frac{[\text{oxidised form}]}{[\text{reduced form}]}$$

$$-0.244 = -0.22 + \frac{0.059}{2} \log \frac{[\text{oxidised form}]}{[\text{reduced form}]},$$

which gives a cysteine to cystine ratio of 7:1. This ratio would be expected to be higher than the GSH:GSSG ratio because of the higher standard reduction potential of the cysteine: cystine redox pair. By dropping the pH to pH 6.00, the standard reduction potential of the system increases by a value of 0.059 V (Segal, 1976), to -0.161 V. Recalculation of the cysteine:cystine ratio needed to maintain the reduction potential of -0.244 V gives a cysteine: cystine ratio of 651:1. Thus, changes in pH have profound effects on the redox potential within a system.

As described by Butor *et al.* (1995), the late endosomal pH may fluctuate from pH 4.5 to alkaline pH values. These fluctuations could possibly induce pH dependent changes in the redox potential. At a fixed concentration and ratio of cysteine:cystine, the reductive potential of a system would tend to be more oxidising at a lower pH than at a higher pH. This would have an immediate effect on the ability of the system to reduce the disulfide bridges of a substrate. This effect might, however, be offset by the denaturing changes induced in the structure of the substrate by low pH. A further effect that these changes would have is on the activation of cysteine proteases, which might be more readily activated at neutral rather than acidic pH values.

In this chapter, exploration of the reductive activation of cathepsin B under various pH conditions is reported. An attempt was made at determining the optimal redox potential for cathepsin B in order to give some indication of the endosomal redox environment in which the enzyme operates. It was found, for example, that the optimal *in vitro* GSH:GSSG ratio for PDI (Lyles and Gilbert, 1991) approximated the GSH:GSSG ratio in the ER (Hwang *et al.*, 1992). Finally, the activation of the enzyme was compared to other proteases to determine whether the observed reductive activation behaviour was unique to cathepsin B.

4.2 Construction of redox buffer/activators

Thiol oxidation occurs extremely rapidly. It has been shown, for example, that the plasma redox status changes markedly within 10-20 s of sampling (Mansoor *et al.*, 1992). These changes cannot be prevented by EDTA or antioxidants (Akerboom and Sies, 1981; Mansoor *et al.*, 1992). These observations are often not considered when describing the role of thiols in supporting proteolysis (Kooistra *et al.*, 1982; Collins *et al.*, 1991). During this project several methods were evaluated that allowed for the deoxygenation of solutions including nitrogen sparging and photo-oxidation of anthroquinone sulfonate. The method chosen was one that is used for the isolation of oxygen sensitive enzymes (Dr Thomas Urbig, pers. comm.).

4.2.1 Reagents and Procedure

Reagents. Oxygen-free nitrogen was obtained from Afrox (South Africa). The reducing agents cysteine, GSH, NADPH, dithiothreitol (DTT), and the disulfide cystine were obtained from Sigma (St. Louis, MO) and were of the highest purity commercially available. All buffers were constructed using Ultrapure Milli-Q water.

Acetate-MES-Tris (AMT) buffers [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA]. Glacial acetic acid (1.43 ml), MES (4.88 g), Tris (6.06 g) and Na_2EDTA (0.375 g) were dissolved in 200 ml of dist.H_2O. When needed, aliquots (20 ml) of solution

were deoxygenated by boiling, cooled under oxygen-free nitrogen, and degassed under vacuum. Reducing agent, or its oxidised counterpart, was added to these solutions at a concentration to accommodate dilution during the assays employed. The pH of each solution was titrated to values in the range 4-8.5 using HCl or NaOH, and the buffers made up to 25 ml. All steps, including the pH and volume adjustments, were done under nitrogen.

4.3 Activation of cathepsin B by various reducing agents

Five reducing agents were tested for their ability to activate bovine cathepsin B: cysteine, cysteamine, GSH, NADPH, and DTT (Fig. 15). Cysteine is believed to be the principal reducing agent within the "lysosome" (Pisoni *et al.*, 1990), and is also the predominant thiol present within human plasma (Mansoor *et al.*, 1992). Cysteamine is the decarboxylated form of cysteine and is recognised by the lysosomal cysteine transporter. Cysteamine is used in the treatment of cystinosis. Cysteamine reacts with cystine to form cysteine and a mixed disulfide of cysteamine and cysteine that is transported out of the lysosome (Pisoni and Thoene, 1991). GSH is the predominant cytosolic reducing agent, and NADPH is responsible for generating the reducing power within the cell. DTT is a highly effective reducing agent that is often used to activate cysteine proteinases *in vitro*. Oxidation of DTT results in a stable cyclic disulfide product which draws the reaction to completion, making DTT a very effective reducing agent. The thiol concentrations used in this assay ranged from 5-100 mM. At first glance these concentrations appear high, but it has been shown that cathepsin L is maximally activated at 200 mM cysteine (Dehrmann, 1998).

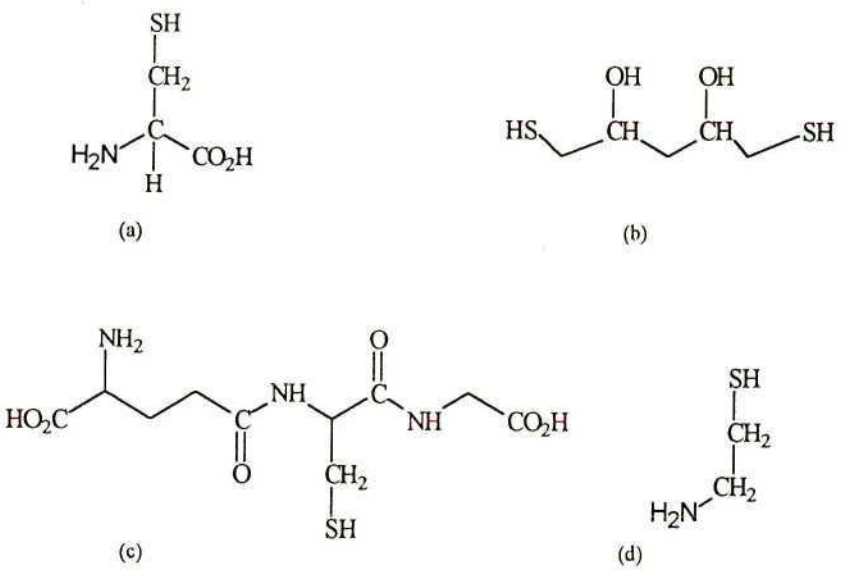

Figure 15. The structures of the thiol-reducing agents used to activate cathepsin B.

(a) Cysteine; (b) DTT; (c) GSH, and (d) cysteamine.

4.3.1 Reagents

Buffer/Activator [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 5-100 mM reducing agent, pH 6.0]. The buffer/activator was made up as described in Section 4.2.1 using the reducing agents cysteine, GSH, NADH, NADPH, DTT and cysteamine.

1 mM CBz-R-R-AMC substrate stock solution. CBz-R-R-AMC (3.1 mg) was dissolved in DMSO (5 ml), divided into 100 µl aliquots and stored at -4°C. When required, this stock was diluted in dist.H_2O to a working concentration of 40 µM .

1 mM CBz-F-R-AMC substrate stock solution. CBz-F-R-AMC (1 mg) was dissolved in DMSO (1.5 ml), divided into 100 µl aliquots. When required, this stock was diluted in dist.H_2O to give a working concentration of 20 µM.

Diluent [0.1% Brij]. Brij (0.1 g) was dissolved in 95 ml of distilled water and made up to 100 ml.

4.3.2 Procedure

Enzyme sample (2.68 µg) was diluted to 125 µl with 0.1% Brij, and buffer/activator (750 µl) added in. This sample was pre-incubated for 2 min at 37°C, and CBz-R-R-AMC or CBz-F-R-AMC substrate solution was mixed in. The rate of change due to liberated AMC was determined continuously in a temperature-controlled Hitachi F-2000 spectrofluorometer with excitation at 370 nm and emission at 460 nm.

4.3.3 Results and discussion

Despite having a lower standard reduction potential than cysteine (Segal, 1976), the tripeptide GSH was not as effective at activating cathepsin B at low thiol concentrations (Fig. 16). However, at a concentration of 100 mM, GSH proved to be the most effective reducing agent tested. Brunk *et al.* (1995) have demonstrated that leakage of lysosomal enzymes due to sub-lethal oxidative stress might be a common event. The inability of GSH to effectively activate cathepsin B at physiological concentrations (Meister, 1995) may offer the cell protection against such lysosomal leakage.

A further indication that standard reduction potentials *per se* do not necessarily correspond to the ability to activate cathepsin B, comes from the results of the activation of the enzyme by NADPH. NADPH, which has one of the strongest reduction potentials within the cell (Segal, 1976), was unable to activate cathepsin B.

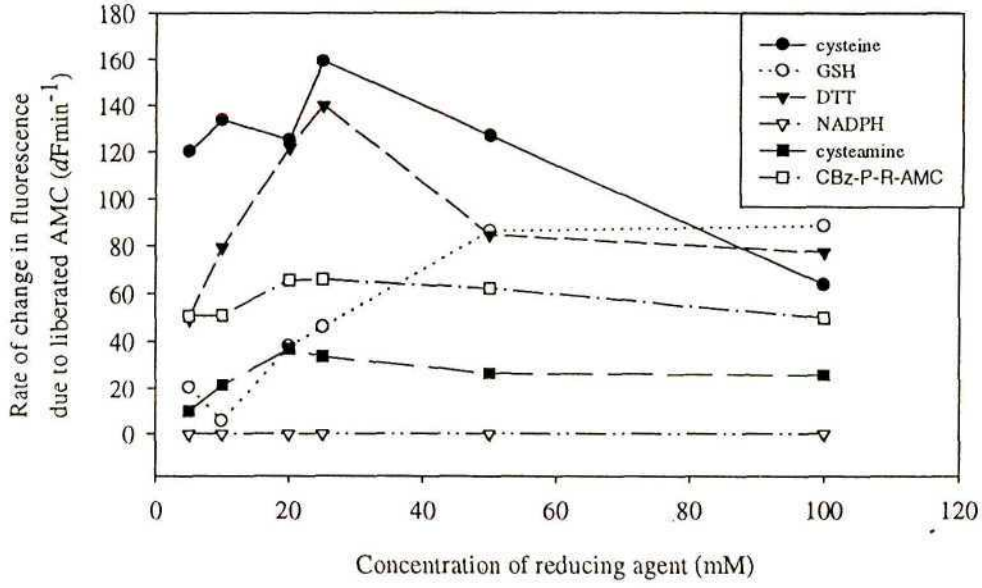

Figure 16. The activation of cathepsin B activity by various reducing agents.

As described in Section 4.3.2, the ability of the reducing agents cysteine (•), GSH (◦), DTT (▼), NADPH (∇), and cysteamine (■) to activate cathepsin B was assessed by measuring the rate of CBz-R-R-AMC hydrolysis. The rate of hydrolysis of the substrate CBz-F-R-AMC by cathepsin B activated with cysteine was also determined (□). The activation conditions were generated using AMT redox buffers (Section 4.2.1) at pH 6.00 and at 37°C. The data represents the mean of 3 determinations.

Cysteamine, the decarboxylated form of cysteine, activated cathepsin B less than cysteine. This was unexpected because of the structural similarities between these molecules. Further, cysteamine activation of cathepsin B showed a steady increase in activation with increasing concentration, with an optimal concentration at 20 mM cysteamine. DTT showed a normal distribution of activity centred around 25 mM DTT. Cysteine activation of cathepsin B, on the other hand, showed a dip in activity at 20 mM cysteine. This unusual pattern of activation was noticed with different batches of enzyme and reducing agent. It was also noted in a previous study (Meinesz, 1996) and hydrolysis of the CBz-F-R-AMC substrate showed a pattern similar to that generated by the CBz-R-R-AMC substrate. Cysteine also proved to be the most effective reducing agent tested, which is consistent with its role as reducing agent within the endosomal system. The effectiveness of cysteine in activating cathepsin B, and its

presence in plasma may have important implications on the enzyme's ability to act extracellularly.

4.4 Determination of the number of cysteine binding sites on cathepsin B

The activation of cathepsin B by cysteine, indicates that the activation of the protease may not be a simple titration of the active site thiol. A simple titration would be expected to show a smooth curve of increasing activity with increasing cysteine concentration up to a maximum, after which activity may decline due to reductive denaturation. Curves showing these activation patterns were obtained for cysteamine and DTT, whilst GSH activation of cathepsin B showed a progressive pattern of increasing activity with increasing GSH concentration.

It was hypothesised that the titration pattern of cathepsin B by cysteine may indicate that there is more than one ligand binding site for cysteine on the enzyme. Thus, cysteine may facilitate inter- and intra-thiol exchanges in cathepsin B. Using a modified method (Tan, 1998) based on Wang-Srivastava plots (Wang and Srivastava, 1994), the number of ligand binding sites for cysteine on cathepsin B was determined. This method graphically describes the number of ligand binding sites by plotting the values $1/v[L]^n$ against the concentration of cysteine [L], where v is the initial rate of the reaction , L is the concentration of cysteine, and n = 0, 1, 2, 3... represents the number of putative ligand binding sites. The number of ligand binding sites is indicated by the first plot showing a non-positive slope when compared to the preceding and succeeding plots (Tan, 1998).

4.4.1 Reagents

Buffer/Activator [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1-100 mM cysteine, pH 5.0-7.0]. The buffer/activator was made up as described in Section 4.2.1 using the reducing agent cysteine at concentrations between 1-100 mM [0.00404 g-0.404 g/20 ml] to accommodate dilution in the assay.

1 mM CBz-R-R-AMC substrate stock solution. This solution was made up as described in Section 4.3.1.

Diluent [0.1% Brij]. This solution was made up as described in Section 4.3.1.

Stop solution [100 mM monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3]. Monochloroacetate (9.45 g), Na-acetate (4.08 g) and glacial acetic acid (4 ml) were dissolved in 950 ml of dist.H_2O, titrated to pH 4.3 with NaOH and made up to 1 litre.

4.4.2 Procedure

Enzyme sample (2.68 µg) was diluted to 125 µl with 0.1% Brij, and 750 µl of buffer/activator added in and the sample was pre-incubated for 2 min at 37°C. CBz-R-R-AMC (125 µl) substrate solution was added in and the reaction was allowed to proceed for 10 min at 37°C. The reaction was terminated by the addition of stop solution (500 µl) and the mixture was kept on ice. The fluorescence of the samples, due to liberated AMC, was determined in a Hitachi F-2000 spectrofluorometer, with excitation at 370 nm and emission at 460 nm.

4.4.3 Results and discussion

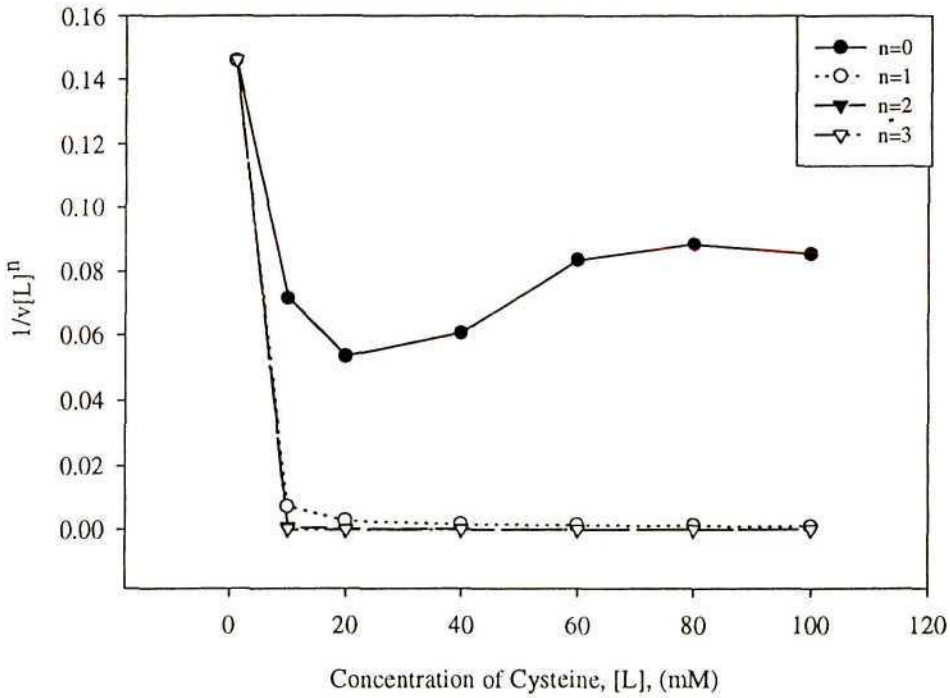

Figure 17. Determination of the number of ligand binding sites for cysteine on cathepsin B at pH 7.0.

The graphical analysis method of Tan (1998) was used to determine the number of ligand binding sites for cysteine on cathepsin B. The first slope showing a zero gradient at reasonably high cysteine concentrations is the curve where n = 1, indicating that there is a single cysteine binding site on cathepsin B. Each data point represents the mean of 3 determinations.

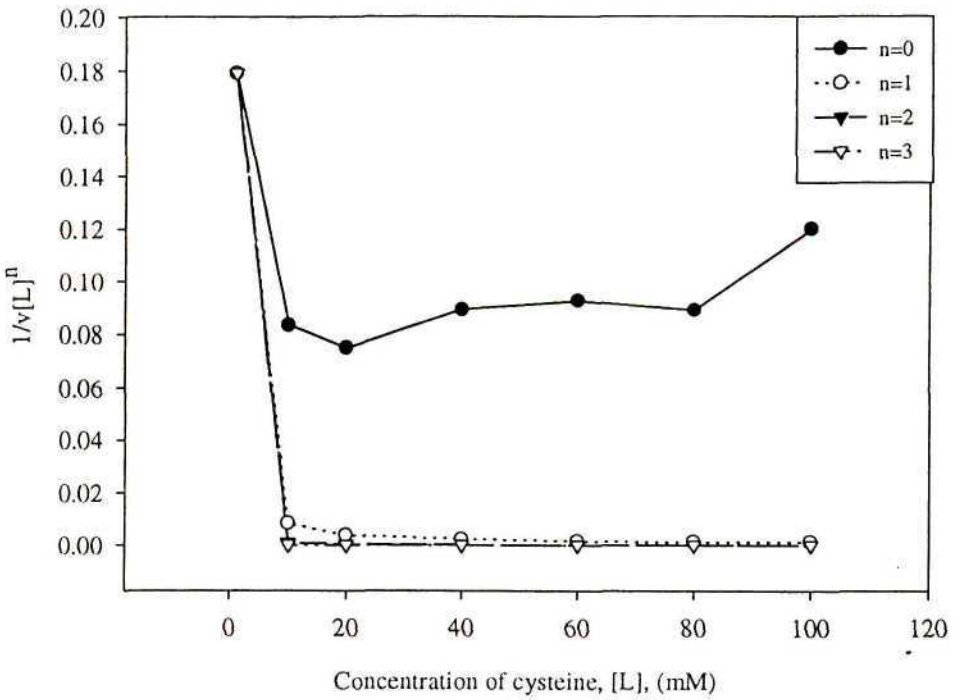

Figure 18. Determination of the number of ligand binding sites for cysteine on cathepsin B at pH 6.0.

The graphical analysis method of Tan (1998) was used to determine the number of ligand binding sites for cysteine on cathepsin B. The first slope showing a zero gradient at reasonably high cysteine concentrations is the curve where n = 1, indicating that there is a single cysteine binding site on cathepsin B. Each data point represents the mean of 3 determinations.

From the results in Figs. 17-19 , its appears that there is only a single ligand binding site for cysteine on cathepsin B. This site is presumably the active site cysteine. Thus, the unusual pattern of cathepsin B activation by cysteine cannot be due to multiple binding sites. The pattern of activation is made all the more puzzling by the fact that the cysteine analogue cysteamine, does not show a similar activation pattern, nor does the tripeptide GSH, which uses a cysteinyl residue for thiol reactions. The unusual activation pattern of cathepsin B by increasing cysteine concentration must await further study for a complete explanation.

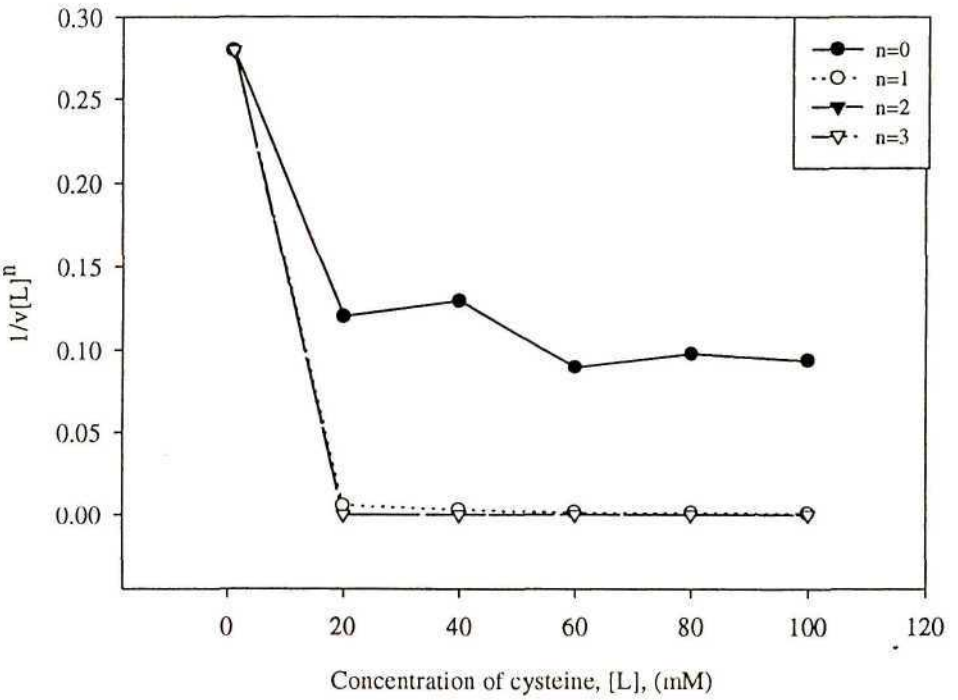

Figure 19. Determination of the number of ligand binding sites for cysteine on cathepsin B at pH 5.0.

The graphical analysis method of Tan (1998) was used to determine the number of ligand binding sites for cysteine on cathepsin B. The first slope showing a zero gradient at a reasonably high cysteine concentration is the curve where n = 1, indicating that there is a single cysteine binding site on cathepsin B. Each data point represents the mean of 3 determinations.

4.5 Effect of pH on the reductive activation of cathepsin B.

The pH optimum and stability of cathepsin B has received much attention because of the enzyme's role in extracellular pathological conditions like cancer and rheumatoid arthritis (Yan *et al.*, 1998). Of interest in the present study was the relationship between the pH optimum of the enzyme and reducing conditions. This relationship may give some indication of the activation conditions for the enzyme along the endocytic pathway. Several groups have described a neutral to slightly alkaline pH optimum for cathepsin B against fluorometric substrates (Willenbrock and Brocklehurst, 1985b; Khouri *et al.*, 1991; Hasnain *et al.*, 1992; Moin *et al.*, 1992; Dehrmann *et al.*, 1996), whilst other groups have described acidic pH optima for the enzyme (Barrett and Kirschke, 1981).

These differences may reflect the unique ability of cathepsin B to behave as both an exopeptidase and an endopeptidase. Most lysosomal cysteine proteases tend to be

endoproteolytic. The occluding loop, which is a unique structural feature on cathepsin B, has two His residues that become protonated at pH 5.5. This allows the active site to dock the carboxy terminus of a substrate, and therefore behave as an exopeptidase (Musil *et al.*, 1991; Illy *et al.*, 1997). As described by Dehrmann *et al.* (1995) many reports describing pH optima for thiol-dependent cathepsins have used anionic buffers of constant molarity over a wide pH range. By doing this there is an increase in ionic strength with an increase in pH, and this was shown to depress cathepsin L activity at higher pH's. This would also apply to cathepsin B.

Dehrmann *et al.* (1996) have also described the increased stability of cathepsin B in extracellular conditions upon reductive activation. This effect was possibly due to the ionic interactions between the active site thiolate-imidazolium ion pair, that stabilises the structure of the enzyme (Turk *et al.*, 1994). Reductive activation of cathepsin B would be expected to be more effective at higher pH's than lower pH for a given concentration of cysteine, if activation depended solely on redox potential (Section 4.1). In this study the activity of the enzyme was probed using thiol concentrations between 10-100 mM over a pH range 4-8.

4.5.1 Reagents

Buffer/Activator [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 10-200 mM cysteine, pH 4.0-8.0]. The buffer/activator was made up as described in Section 4.2.1 between pH 4.0-8.0 using cysteine at concentrations between 10-200 mM [0.0404 g-0.808 g/200 ml].

1 mM CBz-R-R-AMC substrate stock solution. This solution was made up as described in Section 4.3.1.

4.5.2 Procedure

Cathepsin B (0.67 µg) and redox buffer/activator (75 µl) was added to the wells of a white Fluoronunc maxisorp microtitre plate and preincubated for 2 min at 37°C. Thereafter CBz-R-R-AMC substrate (40 µl) was mixed in and the reaction allowed to proceed for 5 min at 37°C. The fluorescence of liberated AMC was determined semi-continously in a fluorescence microplate reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm.

4.5.3 Results and discussion

As can be seen in Fig. 20, the pH optimum for bovine liver cathepsin B varies markedly with changes in the cysteine concentration. The pH optimum of the enzyme appears to be relatively basic at lower thiol concentrations, and relatively acidic at higher cysteine

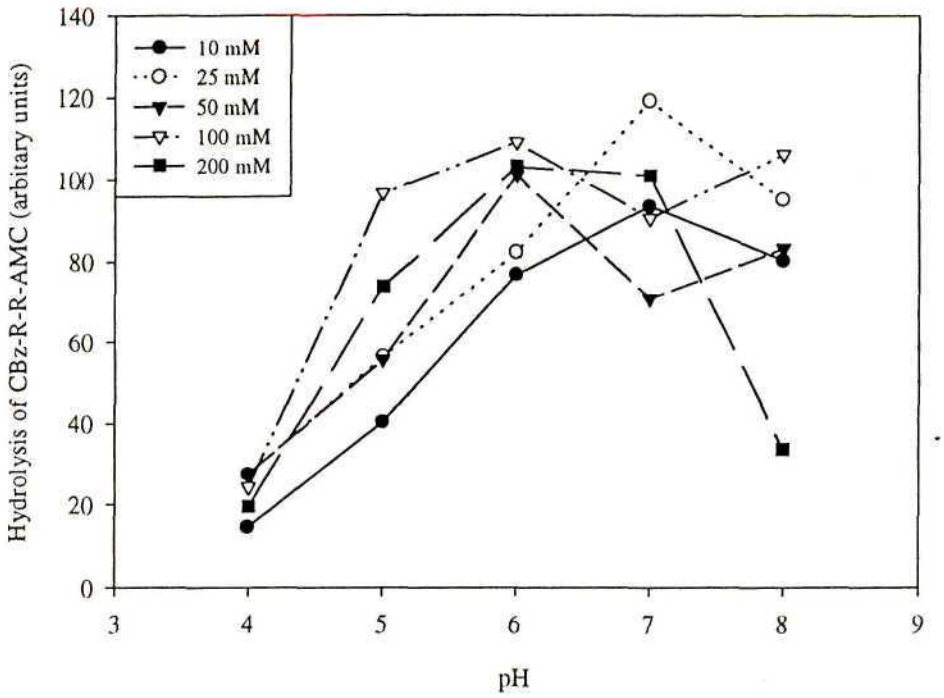

Figure 20. The effect of pH and cysteine on cathepsin B activation.

AMT redox buffers (Section 4.2.1) were constructed with various concentrations of cysteine (0-200 mM) and at various pH values (pH 4-8). The activity of cathepsin B in these buffers was measured as described in Section 4.5.2. The inset describes the relevant cysteine concentrations used. The data represents the mean of 8 determinations.

concentrations. For example, in the presence of 25 mM cysteine, the optimal pH for cathepsin B is pH 7, whilst at 100 mM cysteine, the pH optimum is between pH 5-6. These results are consistent with the relationship between standard reduction potential and pH. As conditions become more acidic, the standard reduction potential increases, requiring more thiol to activate cathepsin B. These results suggest that there might be an optimal standard reduction potential for cathepsin B. However these results must be viewed with caution as the cathepsin B becomes oxidised during isolation. The *in vivo* redox status of the enzyme has not been determined. An attempt was made at determining an optimal potential is described in Section 4.6.

These results also suggest that it might be more thermodynamically favourable to activate (oxidised) cathepsin B at a higher pH than at a lower pH for a given concentration of

cysteine. For example if the intra-"lysosomal" cysteine concentration is 10 mM, at pH 7.0 cathepsin B would be more activated than at pH 4.0 (Fig. 20). Another argument in favour of neutral pH activation of cathepsin B, and an alternate explanation of the phenomenon observed in Fig. 20, is that the pKa of the cysteine thiol moiety is 8.3. Thus, at neutral pH a larger portion of cysteine would be in the active thiolate form, favouring reduction when compared to lower pH's. The activation of cathepsin B by a pH-dependent redox potential, does provide a possible mechanism for regulating proteolysis. By acidifying an organelle, at a fixed concentration of cysteine, the redox conditions within that organelle become relatively oxidising. Thus, cysteine-protease dependent proteolysis could be shut-down by acidification.

Whilst cathepsin B is activated very efficiently at neutral pH's, alkaline pH values (pH 8.0 in this case) would tend to denature the enzyme (Fig. 20). Dehrmann *et al.* (1996) described a pH optimum for human liver cathepsin B between pH 7-7.5. However, the half-life of the enzyme at 37°C and pH 7.2 was 245 ± 11 s, but 857 ± 50 s at pH 6.8. Thus, whilst cathepsin B shows a maximal activity at neutral to slightly basic pH values, it is more stable at lower pH values. The activity of the enzyme must therefore be balanced between its activation and destabilisation values. A scheme suggested by Mort and Buttle (1997) is that cathepsin B could use its endopeptidase activity to cleave a substrate into smaller components at neutral to slightly acidic pH values. As the pH decreases below 5.5, the enzyme's exopeptidase activity would come into operation to hydrolyse these smaller components. The activation of cathepsin B at higher pH is in keeping with this scheme.

4.6 The effect of cystine on cathepsin B activation

The results in Section 4.5 suggested that cathepsin B might be optimally activated at a specific reduction potential. The resistance of cathepsin B to non-thiol activation precluded the use of cyclic voltametry to assess this reduction potential. Instead, a cysteine/cystine redox buffer was used between pH 5-7. Cathepsin B activity was assessed against CBz-R-R-AMC, as well as against haemoglobin. These results were compared against the action of the lysosomal aspartic protease cathepsin D.

4.6.1 Reagents

Buffer/Activator A [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1-100 mM cysteine, pH 5.0-7.0]. The buffer/activator was made up as described in Section 4.4.1 using cysteine at concentrations between 1-100 mM.

Buffer/Activator B [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1 mM cystine, 1-100 mM cysteine, pH 5.0-7.0]. The AMT redox buffers were made

up as described in Section 4.2.1 with the addition of 1 mM cystine (0.135 g/200 ml). Cysteine at concentrations between 1-100 mM (Section 4.4.1) was added to 20 ml aliquots of this buffer, the pH adjusted, and the buffer made up to 25 ml. These steps were all done under nitrogen.

1 mM CBz-R-R-AMC substrate stock solution. This solution was made up as described in Section 4.3.1.

Diluent [0.1% Brij]. This solution was made up as described in Section 4.4.1.

Stop solution [100 mM monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3]. This solution was made up as described in Section 4.4.1.

4.6.2 Procedure

Enzyme sample (2.68 µg) was diluted to 125 µl with 0.1% Brij, and either buffer/activator A or buffer/activator B (750 µl) added in. This sample was pre-incubated for 2 min at 37°C. Thereafter, CBz-R-R-AMC substrate solution (125 µl) was added in, and the reaction was allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of stop solution (500 µl) and the solutions kept on ice. The fluorescence of the samples, due to liberated AMC, was determined in a Hitachi F-2000 spectrofluorometer, with excitation at 370 nm and emission at 460 nm.

4.6.3 Results and discussion

Cathepsin B did not show a requirement for a distinct reduction potential over all the pH values tested. As can be seen in Fig. 21-23, cathepsin B was still maximally activated at pH 7.0 > pH 6.0 > pH 5.0 even in the presence of cystine. In Fig. 21, at pH 7.0, the 'cystine-containing' curve follows the 'cysteine-only' curve until 10 mM cysteine. Thereafter, the cystine curve does not vary as much as the cysteine-only curve. These results suggest that in the presence of cystine, the activity of cathepsin B does not appear to vary with increases in cysteine when compared to reactions with only cysteine. At pH 6.0, the cystine-containing reactions were very similar to cysteine-only reactions (Fig. 22). As with the pH 7.0 cystine-containing reactions, there did not appear to much variation of activity with changes in cysteine concentration. The cystine-containing reactions at pH 5.0 appear to resemble the cysteine-only reactions until 20 mM cysteine. Thereafter, the activity of the cysteine containing reactions do not appear to vary with changes in cysteine concentration when compared to the cysteine-only reactions.

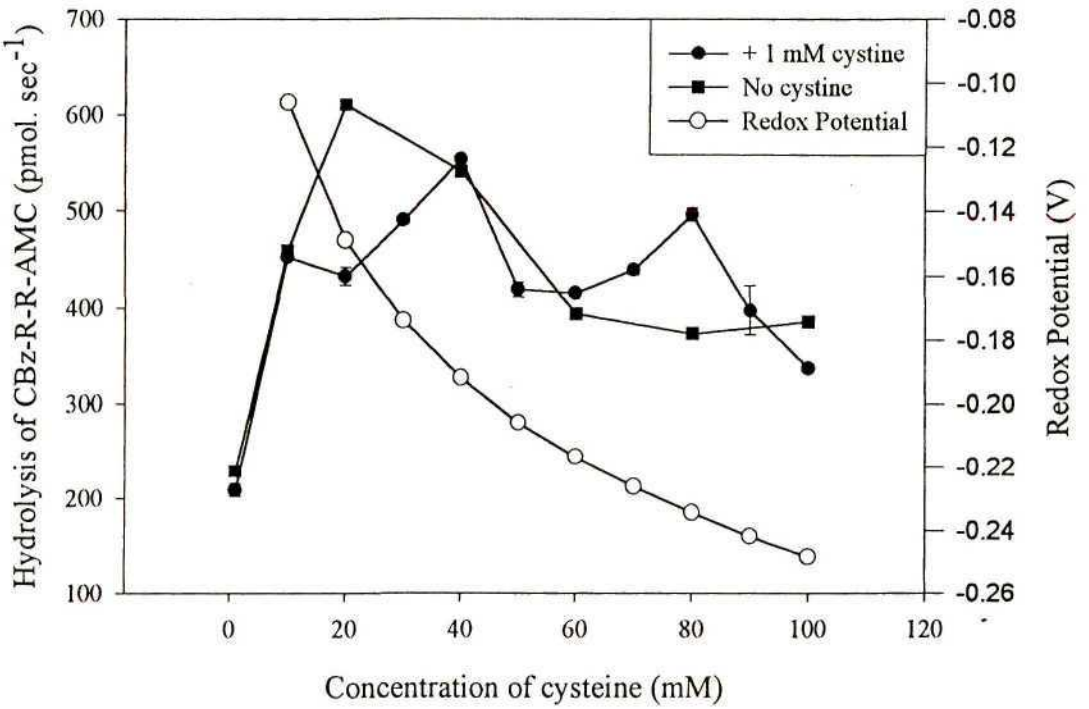

Figure 21. The effect of cystine on cathepsin B activation at pH 7.0.

In order to determine an optimal redox potential for cathepsin B, the enzyme was activated in the presence of 1 mM cystine at various cysteine concentrations, generating cystine:cysteine redox buffers ranging from 1:1 to 1:100. The activation of cathepsin B in the presence of 1 mM cystine (●) was compared to the activation of the enzyme without cystine (■). The data represents the mean ± s.d. (n=3).

Table 4 describes the results of an attempt to determine whether cystine modulates cathepsin B activity such that it is relatively insensitive to changes in cysteine concentration. If it is assumed that the data points for the individual cystine-containing reactions are clustered around a straight line (which represents the modulated activation state of the enzyme), then the standard errors of each line should be significantly smaller when compared to the cysteine-only reactions. The standard errors associated with the cystine-containing reactions are smaller than standard errors associated the cysteine-only reactions (Table 4). Thus it appears that the presence of cystine may regulate cathepsin B activity such that it is relatively insensitive to changes in cysteine concentration. The oxidising ability of cystine could account for this insensitivity to changes in cysteine concentration. However, this effect is not present at lower cysteine concentrations. Further, the effect would be expected to decrease with increasing cysteine concentrations. Since neither of these criteria are met, it can be

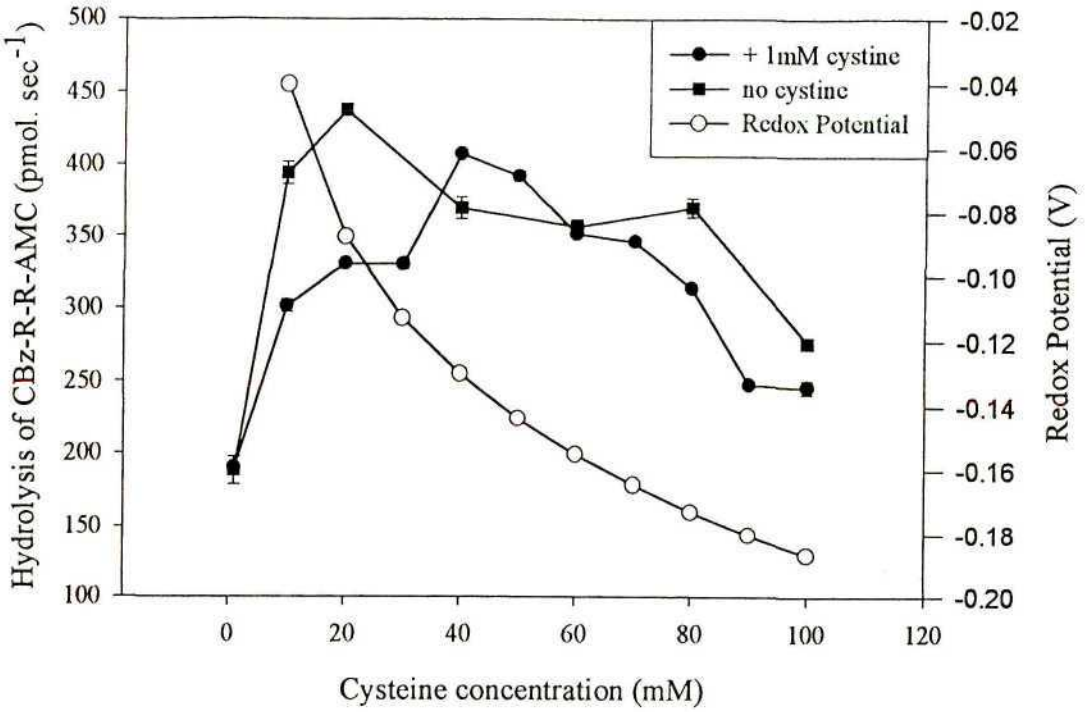

Figure 22. The effect of cystine on cathepsin B activation at pH 6.0.

In order to determine an optimal redox potential for cathepsin B; the enzyme was activated in the presence of 1 mM cystine at various cysteine concentrations, generating cystine:cysteine redox buffers ranging from 1:1 to 1:100. The activation of cathepsin B in the presence of 1 mM cystine (●) was compared to the activation of the enzyme without cystine (■). The data represents the mean ± s.d. (n=3).

Table 4. The ability of cystine to modulate cathepsin B activity.

Reaction	Mean[a] (pmol. sec^{-1})	Standard error (pmol. sec^{-1})
pH 7.0 (+cystine)	421.97	27.34
pH 7.0 (-cystine)	422.07	55.26
pH 6.0 (+cystine)	314.67	19.60
pH 6.0 (-cystine)	333.29	35.80
pH 5.0 (+cystine)	222.34	14.49
pH 5.0 (-cystine)	285.41	36.84

[a] The mean was calculated using all the data points for each reaction. For example, the mean for the pH 7.00 (+cystine) reaction was calculated from the velocities of all the reactions (1-100 mM cysteine, 1 mM cystine) undertaken at pH 7.00 (Fig. 21). The standard error represents the average difference between the actual data points and this mean.

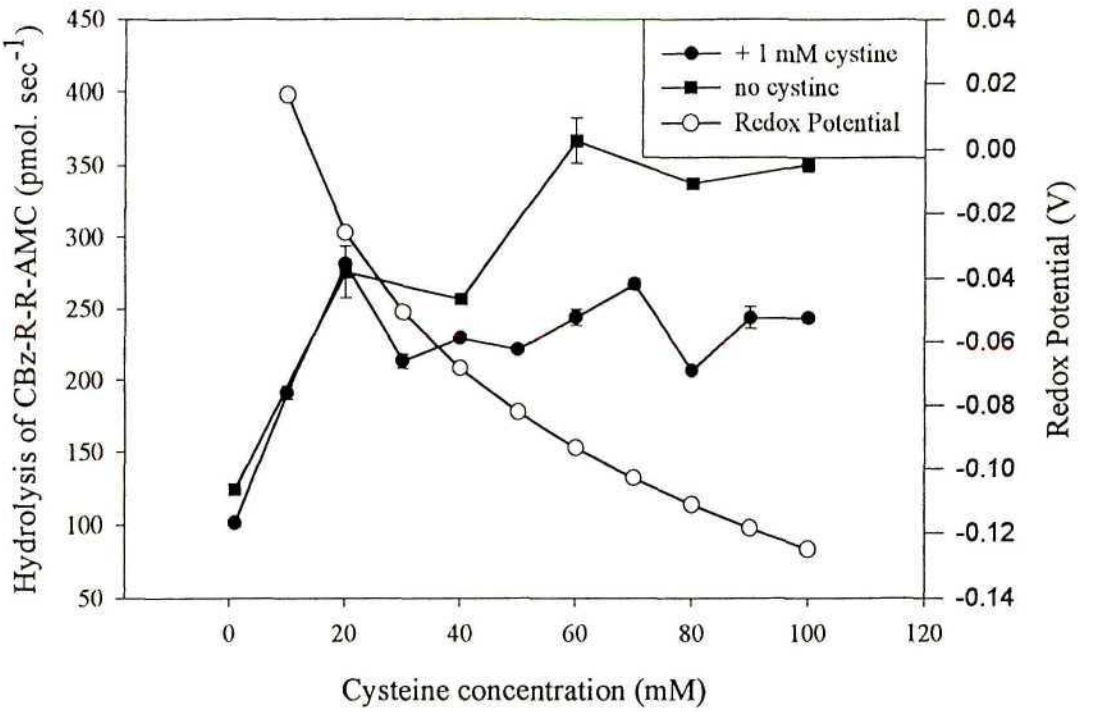

Figure 23. The effect of cystine on cathepsin B activation at pH 5.0.-

In order to determine an optimal redox potential for cathepsin B, the enzyme was activated in the presence of 1 mM cystine at various cysteine concentrations, generating cystine:cysteine redox buffers ranging from 1:1 to 1:100. The activation of cathepsin B in the presence of 1 mM cystine (●) was compared to the activation of the enzyme without cystine (■). The data represents the mean ± s.d. (n=3).

assumed that the modulation of cathepsin B activity is not solely due to the oxidising ability of cystine *per se*. A possible explanation for this "modulating" effect could be that once cathepsin B has been activated specific, or "threshold", redox potential, negative changes in the redox potential no longer affect the activity of the enzyme. The redox potentials for the cystine-containing buffers (Fig. 21-23) were calculated using the Nernst equation. The purported threshold redox potentials for the reactions in Fig. 21-23 were estimated from the graphs to be between -0.02 V to -0.12 V. These redox potential are significantly more oxdising than the relatively oxidising redox environment found within the ER (Hwang *et al.*, 1992). This would allow cathepsin B to operate under relatively oxidising conditions, and was unexpected because the results obtained in Section 4.5 suggest that the enzyme may be regulated by redox potential. The data in Section 4.5 may reflect the activation of an artifactually oxidised enzyme. During isolation the active site thiol could be oxidised to sulfinic acid, and the reduction of this moiety could be reponsible for the pH-thiol response curves in Section 4.5. These results cast doubt on the hypothesised redox regulatory mechanism described in Chapter 1. Thus, an alternate mechanism must exist for the regulation of proteolysis along the endocytic pathway.

4.7 The effect of varying concentrations of cystine on cathepsin B activation.

In order to assess the results in Section 4.6, cathepsin B was activated in the presence of varying concentrations of cystine. Cystine is poorly soluble above 1 mM and the concentration range used varied from 0-1 mM cystine. All the reactions were carried out at a cysteine concentration of 40 mM, which was the optimal cysteine concentration for the activation of cathepsin B in the presence of 1 mM cystine at pH 6.0 and pH 7.0 (Fig. 21-22). Activation of the enzyme was determined between pH 5-7, and the number of ligand binding sites for cystine on cathepsin B was determined as described in Section 4.4.

4.7.1 Reagents

Buffer/Activator A [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 0-1 mM cystine, 40 mM cysteine, pH 5.0-7.0]. The buffer/activator was made up as described in Section 4.2.1 and including cystine at concentrations between 0-1 mM. Cysteine (0.162 g) was added to 20 ml aliquots of this buffer, the pH adjusted, and the buffer made up to 25 ml. These steps were all done under nitrogen.

1 mM CBz-R-R-AMC substrate stock solution. This solution was made up as described in Section 4.3.1.

Diluent [0.1% Brij]. This solution was made up as described in Section 4.3.1.

Stop solution [100 mM monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3]. This solution was made up as described in Section 4.4.1.

4.7.2 Procedure

Enzyme sample (2.68 µg) was diluted to 125 µl with 0.1% Brij, and either buffer/activator (750 µl) added in. This sample was pre-incubated for 2 min at 37°C. Thereafter, CBz-R-R-AMC substrate solution (125 µl) was added in, and the reaction was allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of stop solution (500 µl) and the solutions kept on ice. The fluorescence of the samples, due to liberated AMC, was determined in a Hitachi F-2000 spectrofluorometer, with excitation at 370 nm and emission at 460 nm.

4.7.3 Results and discussion

The most prominent feature of cathepsin B activation, in the presence of varying concentrations of cystine, is the lack of a distinct pattern that would allow cystine to be

classified as an activator or as an inhibitor. This result would be consistent with the results obtained in Section 4.5, which suggested that cathepsin B has an optimal reduction potential. Therefore, cystine would be expected to behave as the oxidation half of a redox system, and not as a classical inhibitor or activator. A further feature of the activation curves presented in Fig. 24 is the variation of the data at pH 7.0>pH 6.0>pH 5.0. As described in Section 4.1, redox potential is a function of pH, and thiol interchange would be favoured at higher, rather than lower, pH values. The increased variation noted at pH 7.0 when compared to the other pH values is probably due to increased thiol interchange at this pH. Similarly thiol interchange would be more thermodynamically favoured at pH 6.0 compared to pH 5.0.

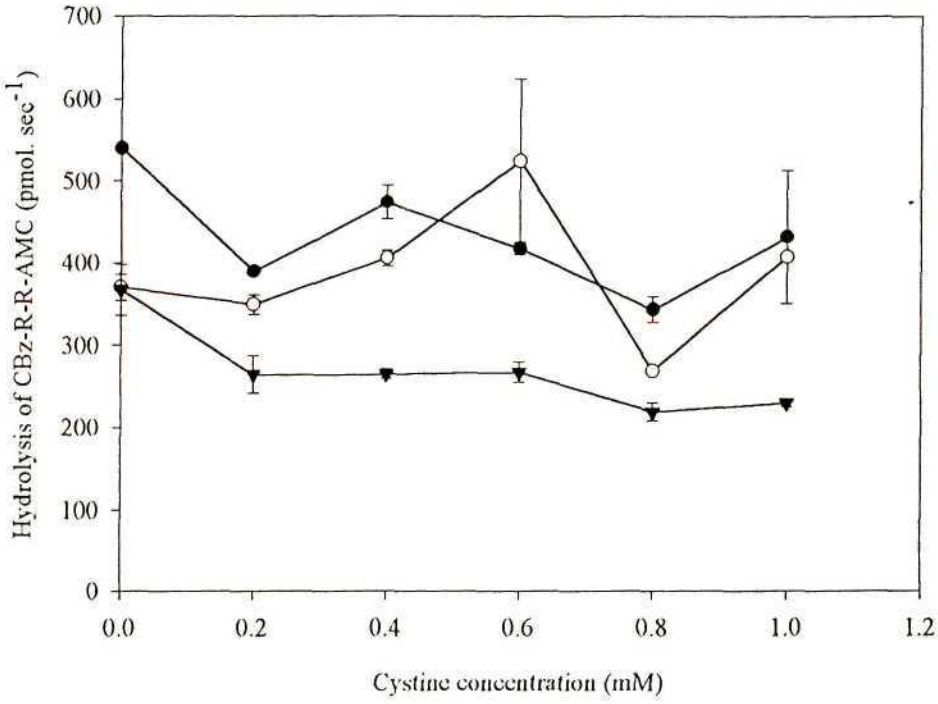

Figure 24. The activation of cathepsin B in the presence of various concentrations of cystine.

Cathepsin B was activated in AMT redox buffers containing varying concentrations of cystine and at 40 mM cysteine (Section 4.7.2). The activation of the enzyme was compared at pH 5.00 (▼), pH 6.00 (○) and pH 7.00 (●). The data represents the mean ± s.d. (n=3).

At first glance, the pH 5.0 data in Fig. 24 seems to indicate that cystine has little or no effect on cathepsin B activation, except for a slight drop in activation as conditions become more oxidising (cystine concentration increases). These results are inconsistent with the effect that 1 mM cystine has on cathepsin B activation with varying cysteine concentrations (Fig. 23). Here cystine, seemed to modulate enzyme activity around an apparently stable velocity. This apparent inconsistency in the results shown in Fig. 23 and Fig. 24 could be explained by

considering the actual ratios of cysteine:cystine. At a concentration of 0.8 mM cystine, in the presence of 40 mM cysteine, the thiol/disulfide ratio is 50:1 and the reaction proceeds at a velocity of 218.93 ± 5.46 pmol. sec^{-1}. From Fig. 24 which describes the activation of cathepsin B in 50 mM cysteine and 1 mM cystine (50:1 thiol disulfide ratio), the activity of the enzyme is very similar (221.41 ± 2.29 pmol. sec^{-1}). The thiol/disulfide ratio given by 0.6 mM in the presence of 40 mM cystine is 67:1. The velocity of this reaction is very similar to the velocity of the cysteine: cystine, 70:1 reaction in Fig. 23. The 0.4 mM cystine: 40 mM cysteine reaction (1/100) does not however correspond to the result in Fig. 23. These results indicate that the activation of the enzyme depends on specific redox potentials, and any pattern in the curves described in Fig. 24 is essentially meaningless. Each reaction has a unique thiol-disulfide generated redox potential, and therefore a distinct velocity. Thus, a distinct pattern of activation may not be apparent.

At pH 6.0, with varying cysteine concentrations, those reactions which included 1 mM cystine showed similar velocities to those reactions without cystine (Fig. 22). In this case, it would have been expected that changes in the cystine concentration would not have large effects on the activation of the enzyme. As the results in Fig. 24 describe, the changing cystine concentration appears to cause large changes in the velocity of the reactions, but not with a specific pattern. A similar fluctuating pattern is observed for the activation of cathepsin B at pH 7.0 with varying concentration of cystine. Unlike the data for pH 5.0, comparing the thiol:disulfide ratios from Fig. 21 and Fig. 22 with Fig. 24 does not give corresponding velocities for enzyme activation at pH 6.0 and pH 7.0. These inconsistencies are possibly due to the increased thiol-exchange and reducing conditions at these higher pH's when compared to pH 5.0. For example, a cystine:cysteine ratio of 1/100, in Fig. 21 for example, is due to a ratio of 1 mM cystine:100 mM cysteine. A 0.4 mM cystine:40 mM cysteine ratio also gives a disulfide:thiol ratio of 1:100, and a similar redox potential, but the conditions in this reaction would be considerably different. Its has been shown by Raman *et al.* (1996) that the absolute concentrations of reactants in a thiol:disulfide reaction influence the redox reactions of that system. In their experiment, a constant thiol:disulfide ratio of 10:1 was maintained for GSH:GSSG, cysteine:cystine, and DTT:oxidised DTT, with varying concentrations of each reagent. It was shown that the refolding of denatured/reduced lysosome was influenced by the absolute concentrations of the reagents in the thiol:disulfide redox buffer.

Apart from the absolute concentration of the redox reagents used, and the pH-mediated changes in redox potential, a further factor that would influence the activity of the enzyme, is the differing half-lives of the enzyme at different pH values. The individual contribution of all these factors to enzyme activity is very difficult to quantify, and thus future experiments to

determine the optimal redox potential of cathepsin B should perhaps focus on structural changes associated with activity rather than activity *per se*.

The data obtained in Fig. 24 was used to determine the number of ligand binding sites for cystine on cathepsin B. As the results in Fig. 25 indicate, cystine does not appear to have any ligand binding sites on cathepsin B. Thus, the activity of the enzyme may be affected by a general redox potential and not by a specific thiol-disulfide interchange at the active site for example. It is therefore reasonable to expect that this potential would affect potential substrates. In order to evaluate the effect that these redox systems would have on cathepsin B activation and proteolysis, the activity of cathepsin B against haemoglobin was evaluated.

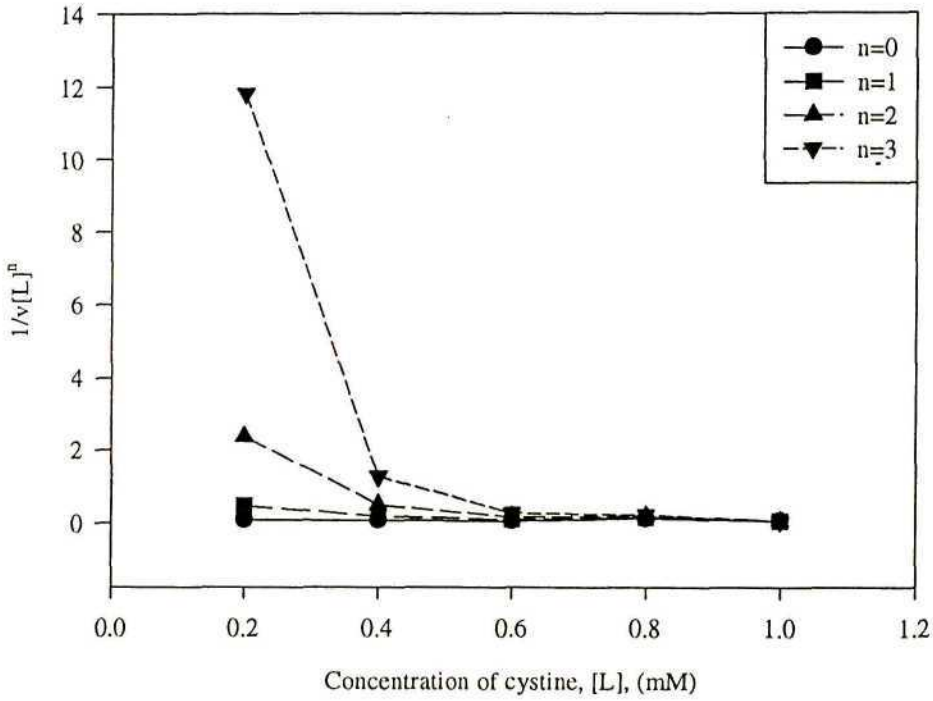

Figure 25. Determination of the number of cystine binding sites on cathepsin B at pH 7.0.

Cathepsin B was activated by 40 mM cysteine in the presence of varying concentrations of cystine. This data was used to generate plots to determine the number of ligand binding sites for cystine on cathepsin B using the method of Tan (1998). As the figure indicates, cystine does not appear to have a specific ligand binding site on cathepsin B. Similar results were obtained at pH 6.0 and pH 5.0 (data not shown).

4.8 Effect of cysteine/cystine on the action of cathepsin B against haemoglobin.

The effect of thiol and disulfide activation on cathepsin B activity was evaluated using an assay based on an assay for cathepsin D. Enzyme sample was incubated with haemoglobin at various concentrations of cysteine and/or cystine between pH 5-7. Hydrolysis of haemoglobin generates TCA-soluble peptides which can be quantified by their absorbance at A_{280}.

4.8.1 Reagents

Buffer/Activator A [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1-50 mM cysteine, pH 5.0-7.0]. The buffer/activator was made up as described in Section 4.6.1.

Buffer/Activator B [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1-50 mM cysteine, 1 mM cystine, pH 5.0-7.0]. The buffer/activator was made up as described in Section 4.6.1.

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

Diluent [0.1% Brij]. This solution was made up as described in Section 4.3.1.

TCA-stop solution [10% (m/v)]. TCA (10 g) was made up to 100 ml with dist.H_2O.

4.8.2 Procedure

Cathepsin B sample (6.7 µg) was diluted to 125 µl with 0.1% Brij, and either buffer/activator A or buffer/activator B (750 µl) added in. This sample was pre-incubated for 2 min at 37°C. Thereafter, haemoglobin substrate solution (150 µl) was added in, and the reaction was allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of TCA-stop solution (500 µl) and kept on ice. The presence of TCA-soluble degradation products was determined at A_{280} using a Pharmacia Ultraspec 2000 spectrophotometer. In control experiments, the enzyme sample was replaced by dist. H_2O in the reaction mix. The TCA-soluble supernatants of these reactions were used as blanks for the corresponding reactions with active enzyme.

4.8.3 Results and discussion

The pattern of hydrolysis of haemoglobin by cathepsin B at various cysteine concentrations (Fig. 26-28) is very different to the patterns observed in Fig. 21-23. Unlike cathepsin B

hydrolysis of CBz-R-R-AMC, haemoglobin hydrolysis does not proceed optimally at pH 7.0 over all concentrations of cysteine. For example, at 20 mM cysteine, the hydrolysis of haemoglobin proceeds optimally at pH 6.0>pH 5.0>pH 7.0. At this concentration of cysteine hydrolysis of the CBz-R-R-AMC substrate proceeds optimally at pH 7.0 >pH 6.0>pH 5.0. The differences observed in the pH optimum could be due to the effect of the haemoglobin substrate. The lower pH might promote acid-dependent denaturation of the haemoglobin substrate, increasing the number of susceptible cleavage sites on the molecule.

An alternate explanation is that these results are artifacts generated by the experimental procedure employed. The degree of haemoglobin hydrolysis is assessed by TCA-soluble peptides. At lower pH's (< pH 5.5), cathepsin B behaves as an exopeptidase (Illy *et al.*, 1997), an activity which is more likely to generate TCA-soluble products, than the endopeptidase activity of the enzyme. Thus, the results obtained at higher pH's (pH 7.0) may be an underestimate of the extent of haemoglobin hydrolysis. One other factor could contribute to the differences observed in hydrolysis of the whole protein when compared to the fluorometric substrate. The hydrolysis of a whole protein by lysosomal proteases is a synergistic process between the enzymes and the reducing effect of a thiol (Kooistra *et al.*, 1982). These factors might work optimally together at specific conditions of pH vs thiol concentration vs enzyme activity. This could explain the variability of the responses to changing thiol concentration (Fig. 26-28).

The most striking aspect of the data presented in Fig. 26-28, is the stimulating effect of cystine on the proteolysis of haemoglobin, an effect not seen with the fluorometric assay. This stimulatory effect could be directed at either cathepsin B or against the haemoglobin substrate. It is unlikely that cathepsin B activation by cystine is responsible for the increased proteolysis seen in Fig. 26-28. Firstly, as the results in Section 4.7 describe, there are no ligand binding sites for cystine on cathepsin B and so it is difficult to imagine how the disulfide might stimulate proteolysis. Also, this stimulatory effect is not observed for cathepsin B hydrolysis of CBz-R-R-AMC. This suggests that cystine has an effect on the haemoglobin substrate. Cystine might therefore be more than just a by-product of lysosomal hydrolysis.

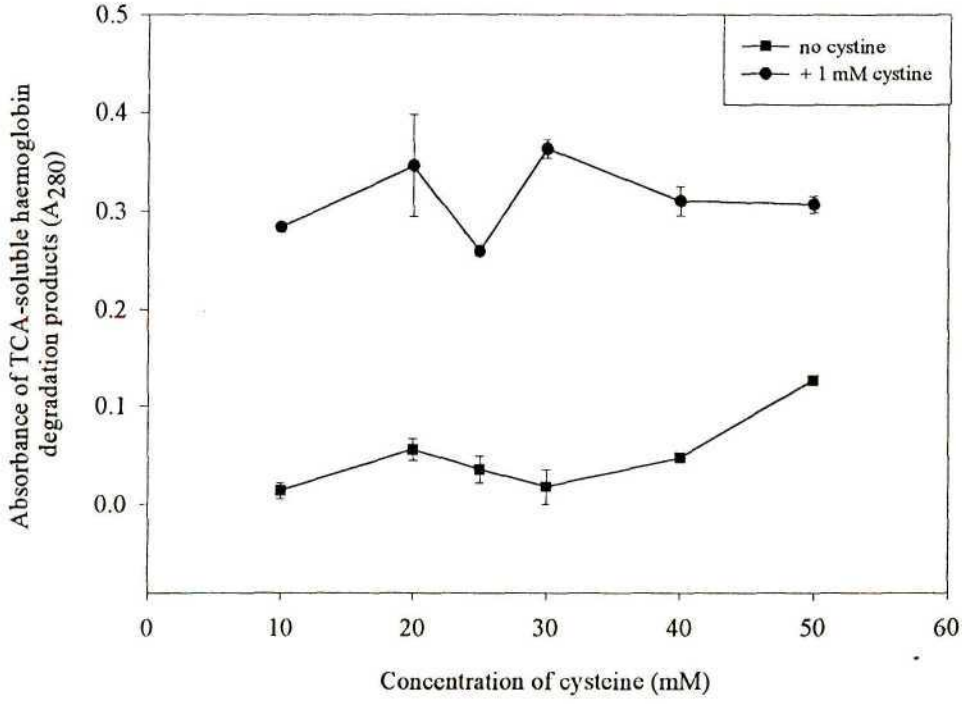

Figure 26. The effect of cystine on the hydrolysis of haemoglobin by cathepsin B at pH 7.0.

In order to determine the effect of cystine on cathepsin B activity against a whole protein substrate, cathepsin B was activated in the presence of 1 mM cystine a various cysteine concentrations (1-50 mM) (●). These reactions were compared to the activity of cathepsin B without cystine (■). The assay is described in Section 4.8.2. The data represents the mean ± s.d. (n=3).

Cystine could stimulate hydrolysis of haemoglobin by increased denaturation of the haemoglobin substrate. However, the activities of cathepsin B described in Fig. 26-28 were relative to controls that included cystine (Section 4.8.2), suggesting that this is not the case. Further, there is no evidence in the literature for this denaturing type of activity. On the contrary, it appears that cystine tends to promote the correct folding of substrates. Raman *et al.* (1996) found that cystine alone (in the absence of cysteine) was able to stimulate the folding of denatured and reduced lysozyme. Similarly GSSG, by itself, was also able to promote folding.

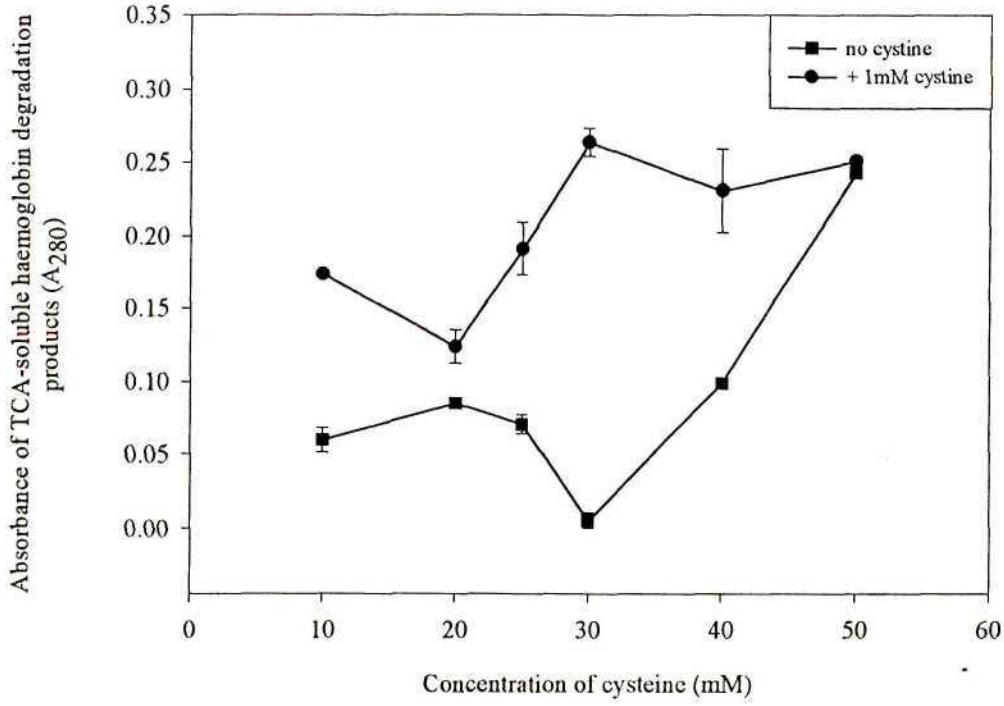

Figure 27. The effect of cystine on the hydrolysis of haemoglobin by cathepsin B at pH 6.0.

In order to determine the effect of cystine on cathepsin B activity against a whole protein substrate, cathepsin B was activated in the presence of 1 mM cystine a various cysteine concentrations (1-50 mM) (●). These reactions were compared to the activity of cathepsin B without cystine (■). The assay is described in Section 4.8.2. The data represents the mean ± s.d. (n=3).

How would this explain the increased proteolysis observed in Fig. 26-28? Consider the hydrolysis of haemoglobin: during this process, the hydrophobic core regions that are normally shielded from the aqueous environment presumably become exposed. These exposed hydrophobic regions could bind to other hydrophobic patches, resulting in aggregation. This insoluble aggregate would be unavailable for hydrolysis, effectively decreasing the substrate concentration. The oxidising ability of cystine to promote protein structure, might decrease the hydrophobic interactions between substrate molecules being degraded. This would increase the available substrate in the system. Support for this interperation of the results above comes from the fact that the reaction velocites of the cysteine-only curves were significantly smaller than those undertaken in the presence of cystine as well. Further, all the reactions described in Fig. 26-28 were undertaken relative to control reactions without enzyme (Section 4.8.2) suggesting that the stimulation of

proteolysis observed with cystine was a synergystic process between disulfide and cathepsin B. This would give wieght to the suggestion that cystine may increase the available substrate within the system. That the stimulatory effect observed in Fig. 26-28 may be due to the oxidative properties of cystine, is evident by the way that the velocities of the cysteine-only, and the cystine-containing reactions approach each other with increasing concentrations of cysteine. As the concentration of cysteine increases, the conditions within the system become more reducing, abrogating the effect of cystine.

Most studies on the relationship between lysosomal enzymes and thiols have assumed that the lysosomal milieu is reducing. This would activate lysosomal enzymes, and stimulate the degradation of the substrate by loosening its conformation. Cystine has been assumed to be a degradation by-product that is exported out of the lysosome by a cystine-specific transporter (Pisoni and Thoene, 1991). The experimental results reported here suggests that cystine stimulates proteolysis of substrate, and might be a functional component of a redox buffer system. This system could prevent aggregation of partially degraded substrate, thereby increasing the substrate available for hydrolysis. This redox system may be functionally similar to the ER GSH:GSSG system. The ER redox system (together with chaperonins) serves to promote the folding of nascent polypeptides, preventing aggregation through hydrophobic interactions (Johnson and Craig, 1997). The endosomal system on the other hand, serves to undo this process, and it is not surprising that, in the complex milieu of the endosomal system, the aggregation of substrate through inappropriate hydrophobic interactions could be an important factor during hydrolysis. It should be noted however that the experimental data presented here does not give a definate mechanism on how cystine might work in stimulating proteolysis. Unlike lysozyme, haemoglobin does not use disulfide bonds to maintain its structure and more work needs to be done to determine the mechanism through which cystine exerts its effects.

Support for this concept comes from the work of Cuervo and Dice (1998). These authors have found that a constitutively expressed heat shock protein, hsc 73, is selectively internalised into the endosomal system from the cytosol. Hsc 73 appears to bind to a specific cytoplasmic tail sequence of LAMP-2, an integral lysosomal membrane protein, and is internalised through a pore on the lysosomal membrane. Thus the lysosomal-endosomal matrix has 2 components to prevent protein aggregation. The envisioned degradation of a protein within the endosomal system might involve multiple steps of degradation, and shielding of hydrophobic groups by either chaperonins or redox-buffer mediated folding. These steps would ensure that no substrate is lost through aggregation

The redox system within the late-endosomal/lysosomal system is presumably more reducing than the ER redox system. The cysteine:cystine system must promote substrate denaturation, but prevent aggregation. This system appears to be well suited to the endosomal system.

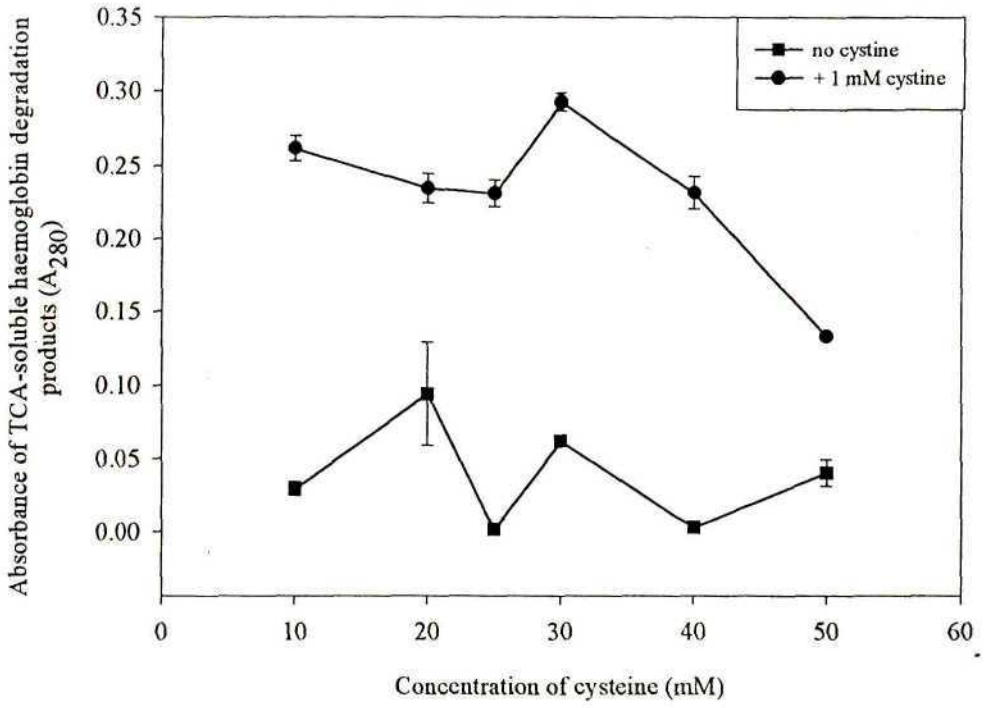

Figure 28. The effect of cystine on the hydrolysis of haemoglobin by cathepsin B at pH 5.0.

In order to determine the effect of cystine on cathepsin B activity against a whole protein substrate, cathepsin B was activated in the presence of 1 mM cystine a various cysteine concentrations (1-100 mM) (●). These reactions were compared to the activity of cathepsin B without cystine (■). The assay is described in Section 4.8.2. The data represents the mean ± s.d. (n=3).

Cysteine is a highly effective activator of lysosomal cathepsin B when compared to GSH for example (Section 4.3). Also, the cysteine:cystine redox pair would not be susceptible to hydrolysis by lysosomal proteases as would a tripeptide like GSH. Despite their similar potentials, the cysteine:cystine system also has a greater capacity for folding relatively high concentrations of substrate when compared to the GSH:GSSG system (Raman *et al.*, 1996).

An argument against such a system would be that the rate of proteolysis is sufficiently rapid to prevent the hydrophobic interactions responsible for aggregation. It has been shown that protein turnover within the 'lysosome' occurs with a half-life of approximately 8 min. This rapid rate is probably due to the high concentration of proteases within the system, which can approach 1 mM for certain enzymes (Bohley and Seglen, 1992). However, aggregation of substrate can occur very rapidly. For example, denatured/reduced hen lysozyme, at concentrations from 148 µg/ml to 18.5 µg/ml, aggregates completely in less than 1 minute

(Goldberg *et al.*, 1991). An appropriate redox system, and chaperonins if necessary, might prevent such aggregation.

4.9 Cathepsin D hydrolysis of haemoglobin in the presence of cysteine/cystine redox buffers.

In order to determine whether cystine exerts a general effect on lysosomal proteolysis, its effect on the hydrolysis of haemoglobin by cathepsin D was assessed. If the conclusions described in Section 4.8 are accurate, cystine would be expected to promote proteolysis by cathepsin D. Such a result would give further weight to the suggestion that cystine exerts its effect on the substrate rather than on the cathepsin B enzyme.

4.9.1 Reagents

Assay buffer A [250 mM sodium citrate, 0.02% NaN_3, 0-50 mM cysteine, 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. Cysteine (0-0.202 g) was added to aliquots (20 ml) of this buffer to give a final concentrations after dilution of between 0-50 mM. After the addition of cysteine, the pH was checked and the solution made up to 25 ml. All steps were carried out under nitrogen.

Assay buffer B [250 mM sodium citrate, 0.02% NaN_3, 1 mM cystine, 0-50 mM cysteine pH 3.2]. Citric acid (26.3 g), NaN_3 (0.1 g), and cystine (0.3375 g) were dissolved in about 450 ml of dist.H_2O, adjusted to pH 3.2 with NaOH and made up to 500 ml.. Cysteine (0-0.202 g) was added to aliquots (20 ml) of this buffer to give final concentrations (after dilution during the assay) between 0-50 mM. After the addition of cysteine, the pH was checked and the solution made up to 25 ml. All steps were carried out under nitrogen

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

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

4.9.2 Procedure

Cathepsin D sample (6.7 µg) was diluted to 125 µl with 0.1% Brij, and either assay buffer A or assay buffer B (750 µl) added in. Samples were pre-incubated for 2 min at 37°C. Thereafter, haemoglobin substrate solution (150 µl) was added in, and the reaction allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of TCA-stop solution (500 µl) and kept on ice. The presence of TCA-soluble degradation products was determined

at A_{280} using a Pharmacia Ultraspec 2000 spectrophotometer. In control experiments, the enzyme sample was replaced by dist. H_2O in the reaction mix. The TCA-soluble supernatants of these reactions were used as blanks for the corresponding reactions with active enzyme.

4.9.3 Results and discussion

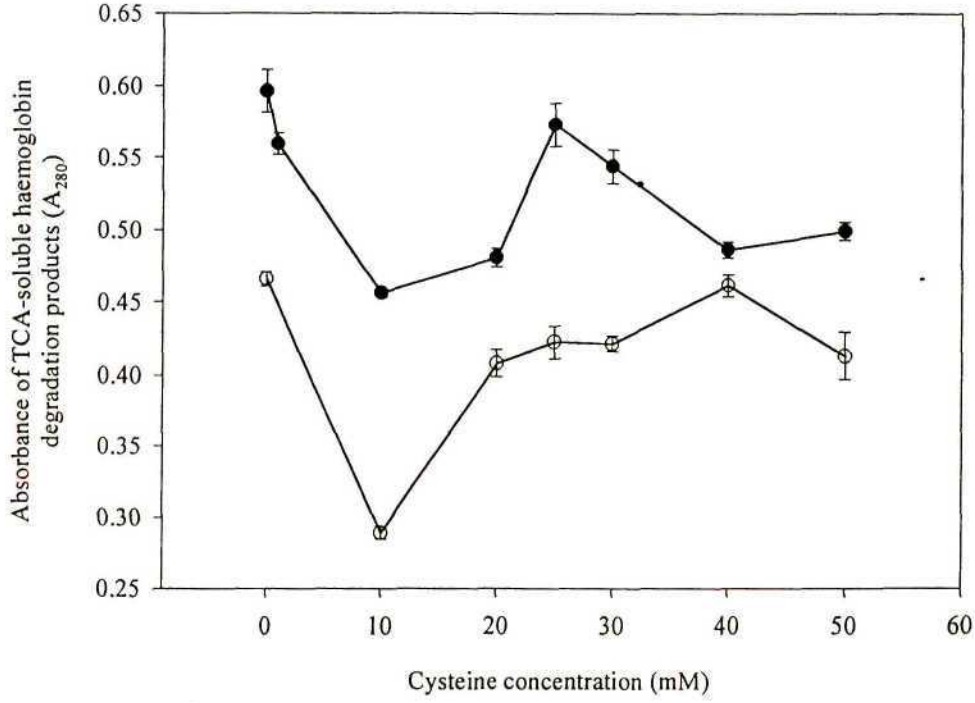

Figure 29. The effect of cysteine and cystine on cathepsin D hydrolysis of haemoglobin.

Cathepsin D was activated in the presence of cysteine (1-50 mM) (O). These reactions were compared to reactions that included 1 mM cystine, also in the presence of cysteine (1-50 mM) (●). The assay was undertaken at pH 3.2 and is described in Section 4.9.2.

Cystine seems to promote proteolysis of haemoglobin by cathepsin D at all concentrations of cysteine tested (Fig. 29). Thus, the effect of the disulfide is not limited to cysteine proteinase mediated hydrolysis. Cystine stimulation of haemoglobin proteolysis occurs synergystically with cathepsin D (see "control reactions", Section 4.9.2). The curves in Fig. 29 show a similar response to changes in cysteine concentration, and approach each other as the cysteine concentration increases. This pattern, similar to that observed in Section 4.8, suggests that stimulation of proteolysis is due to the oxidative properties of cystine. In fact, maximal

activation of proteolysis occurs in the absence of cysteine and at a cystine concentration of 1 mM. Both curves show a local minimum at 10 mM cysteine. The repression of cathepsin D activity at 10 mM cysteine has been reported previously, although no explanation for this effect has been offered (Ikeda *et al.*, 1990).

Both the cysteine-only and the cystine-containing reactions show enhanced proteolysis at cysteine concentrations above 10 mM. Because this effect is observed in both curves, it probably represents the denaturation of the haemoglobin molecule. These results would be consistent with those reported by Kooistra *et al.* (1982) and Mego (1984).

4.10 The effect of cysteine on the action of papain and trypsin.

The rate-limiting steps in the hydrolysis of a protein substrate, along the endocytic pathway, are probably carried out by endoproteases (Bohley and Seglen, 1992). Endoproteases would generate polypeptide fragments that can then be hydrolysed more effectively by the lysosomal exopeptidase pool. The majority of lysosomal endoproteases are cysteine proteases, with four exceptions. Cathepsins D and E are aspartic proteases with acidic pH optima. Their role is presumably to process substrate under low pH conditions, where the cysteine proteases might be inefficiently activated (Section 4.5). The endosomal system also has 2 serine endoproteases, cathepsin G and prolyl endopeptidase (Bohley and Seglan, 1992). Cathepsin G expression is confined to haematopoietic cells, but prolyl endopeptidase is more widely expressed. Serine proteases are widely distributed and expressed in mammalian tissues (Barrett and McDonald, 1980). These proteases usually have neutral to slightly alkaline pH optima and, unlike cysteine proteases, do not require reducing conditions for activation. Despite their wide expression, pH optima and activation conditions, serine endoproteases have not been selected for in the endosomal-lysosomal system. Cysteine proteases, which are not as commonly expressed in mammalian tissues, appear to fill the role of endoproteolytic hydrolysis within the endosomal system.

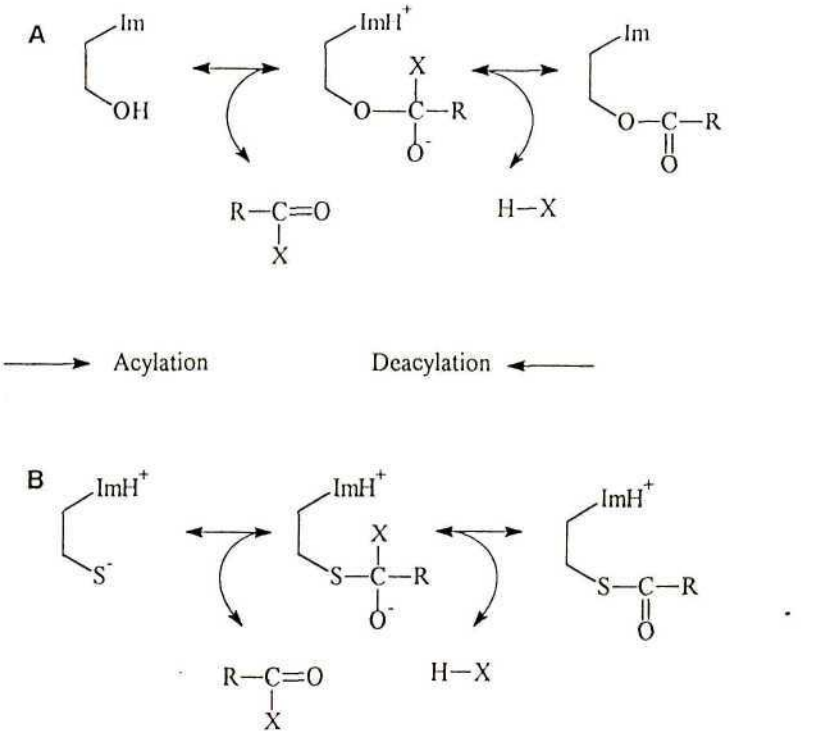

Figure 30. Scheme of the mechanism of action of serine (A) and cysteine (B) proteases.

X stands for OR' or NHR' group in acylation and for OH group in deacylation (Polgar *et al.*, 1986).

An important reason why cysteine proteases would be used in preference to serine proteases, is that they tend to be more efficient catalysts. The difference in their catalytic efficiency is based on the mechanism of proteolysis by the active site residues (Fig. 30). Serine protease mediated cleavage of a substrate involves general base-catalysed acylation, resulting in the formation of a tetrahedral intermediate (THI). The THI undergoes general acid catalysed decomposition and, in a symmetrical process, deacylation. The first step in deacylation is the general base catalysed formation of a second THI, and this is followed by general acid based decomposition of this THI. Cysteine protease catalysis also involves acylation and deacylation steps, but these steps involve simple nucleophilic attack by the active site cysteine. Thus, proteolysis by cysteine proteases tends to be simpler and more efficient (Polgar *et al.*, 1986).

There are two further mechanistic differences between the action of serine and cysteine proteases. Firstly cysteine proteases, which have a thiolate-imidazolium active site, lack the catalytic triad present on serine proteases (serine, histidine and aspartic acid). The aspartate is responsible for charge stabilisation of the negative THI and positive imidazolium ion

generated during catalysis. Charge stabilisation of this nature does not play a role in the cysteine protease hydrolytic mechanism. A further difference between the two classes of protease, is that it is critical for serine proteases to stabilise the oxyanion of the THI (by hydrogen bonding); a requirement not essential for cysteine protease hydrolysis (Polgar *et al.*, 1986).

The dependence of serine proteases on the catalytic triad, and on oxyanion stabilisation, is due to the energy required to activate serine proteases from their ground state to the active transition state, i.e. from the neutral to ion-pair form of the enzyme. Cysteine proteases do not require this investment of energy as the thiolate imidazolium ion pair is already 'activated' and there is no need for charge separation. Another mechanistic difference between these two classes of protease is that the thiolate has a more acidic pK_a than the hydroxyl group and this favours the formation of the thiolate-imidazolium ion pair. This ion pair makes the thiol group an extremely good nucleophile for acylation, and also a good leaving group in deacylation. These factors together ensure that transitional state stabilisation, which determines the thermodynamic and kinetic parameters of catalysis, is realised in a simpler way in cysteine than in serine proteases (Polgar *et al.*, 1986).

The thiolate-imidazolium ion pair has another advantage that would favour the use of cysteine proteases in the endosomal/lysosomal system. Cysteine proteases have a broad pH-activity profile that is due to the existence of 2 ionisation pathways for the active site residues. Consider the acidic limb in Fig. 31: as the pH decreases the thiolate group would become protonated at its pK_a which is approximately pH 4 in papain. This low pK_a is due to the microenvironment surrounding the thiolate. In order for the enzyme to be completely inactivated (neutral form), the pH has to increase to cause ionisation of the imidazole proton. However, increasing the pH would favour the formation of the ion pair. A similar argument applies for the basic limb (Storer and Menard, 1994).

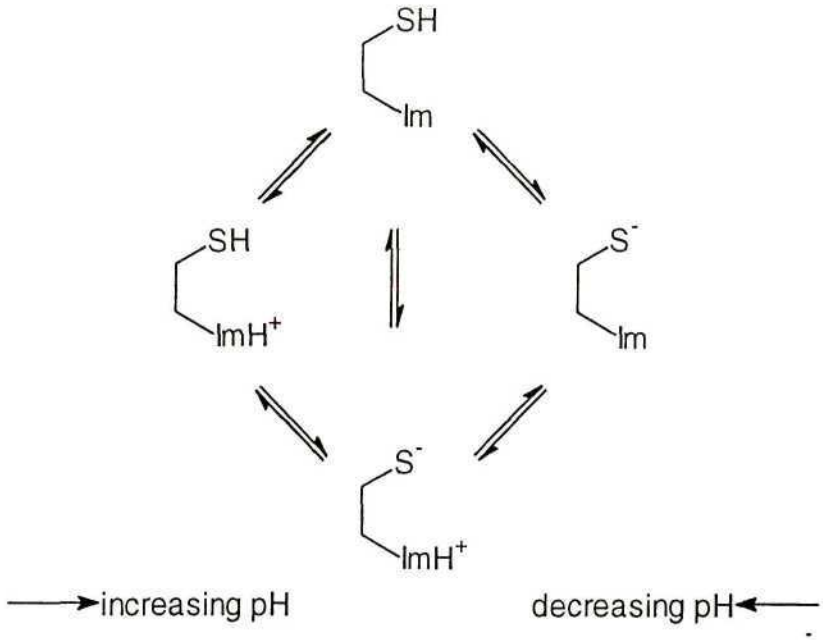

Figure 31. The change in the ion state of the active site residues of cysteine proteases with changes in pH.

The broad substrate specificity of cysteine proteases is due to the stability of the active site thiolate-imidazolium ion pair. Structural features ensure that the ionic forms of the active site residue are favoured over the neutral form. Further, the ion pair itself is resistant to pH-dependent inactivation. For example, as the pH decreases, the active cysteine should become protonated. In order for the enzyme to become the neutral form, the pH must increase, favouring formation of the thiolate-imidazolium ion pair (Storer and Menard, 1994).

This study has yielded results that suggest that cysteine proteases within the endosomal system might be regulated by pH-dependent changes in redox potential within the system (Section 4.5). Thus, proteolysis could be regulated without the need for specific inhibitors. The results obtained in preceding sections suggest one further property that distinguishes cysteine proteases as being useful proteases within the endosomal system. Cathepsin B and cathepsin L (Dehrmann *et al.*, 1995) appear to be stable to high concentrations of reducing agent which may be well in excess of the physiological thiol concentrations (Section 4.5). It was decided to explore whether this is a unique property of cysteine proteases, such as cathepsin B, by comparing the activation of trypsin, a serine protease, with another cysteine protease, papain, with increasing concentrations of reducing agent.

4.10.1 Reagents

Buffer/activator A [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1-50 mM cysteine, pH 6.0-7.0]. The buffer/activator, used to activate papain, was made up as described in Section 4.6.1.

Buffer/activator B [100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 0.02% NaN_3, 1mM cystine, 1-50 mM cysteine, pH 6.0-7.0]. The buffer/activator, used to activate papain, was made up as described in Section 4.6.1.

Trypsin assay buffer [10 mM Tris-HCl, 1-50 mM cysteine, pH 8.00]. Tris (0.061g) was dissolved in dist.H_2O (40 ml), titrated with HCl to pH 8.0 and made up to 50 ml. Cysteine (0-0.202 g) was added to aliquots (20 ml) of this buffer to give final concentrations (after dilution during the assay) of between 0-50 mM. After the addition of cysteine, the pH was checked and the solution made up to 25 ml. All steps were carried out under nitrogen

Papain solution [0.2 mg/ml]. Papain (0.2 mg) was dissolved in buffer/activator (1 ml, Section 3.2.1.1) without the addition of DTT.

Trypsin solution [0.2 mg/ml]. Trypsin (0.2 mg) was dissolved in trypsin diluent (1 ml).

Trypsin diluent [2 mM HCl]. HCl [32% (v/v); ρ = 1.16 $g.ml^{-1}$] (19.6 µl) was dissolved in dist.H_2O (100 ml).

1 mM CBz-R-AMC. CBz-R-AMC (0.9 mg) was dissolved in DMSO (1.5 ml), and stored at 4°C. This stock solution was diluted to a 20 µM working solution with dist.H_2O when required.

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

Diluent [0.1% Brij]. This solution was made up as described in Section 4.3.1.

TCA-stop solution [10% (m/v)]. TCA (10 g) was made up to 100 ml with dist.H_2O.

4.10.2 Procedure

For the synthetic substrate assays, the appropriate assay buffer (75 µl) was added to the enzyme sample (12.5 µl) in a white Fluronunc maxisorp microtitre plate. This mixture was incubated at 37°C for 2 min and thereafter substrate solution (25 µl) was added in. After 10 min at 37°C the fluorescence of the liberated 7-amino-4-methylcoumarin was determined in a fluorescent microplate reader (Cambridge Technology, Model 7620) with excitation at 370 nm and emission at 460 nm. Enzyme activity was described as arbitrary fluorescence

units. For the haemoglobin substrate assays, enzyme (100 µl) and the appropriate assay buffer (750 µl) were pre-incubated for 2 min at 37°C. Thereafter, haemoglobin substrate solution (150 µl) was added in, and the reaction was allowed to proceed for 10 min at 37°C. Reactions were terminated by the addition of TCA-stop solution (500 µl) and kept on ice. The presence of TCA-soluble degradation products was determined at A_{280} using a Pharmacia Ultraspec 2000 spectrophotometer. In control experiments, the enzyme sample was replaced by dist. H_2O in the reaction mix. The TCA-soluble supernatants of these reactions were used as blanks for the corresponding reactions with active enzyme.

4.10.3 Results and discussion

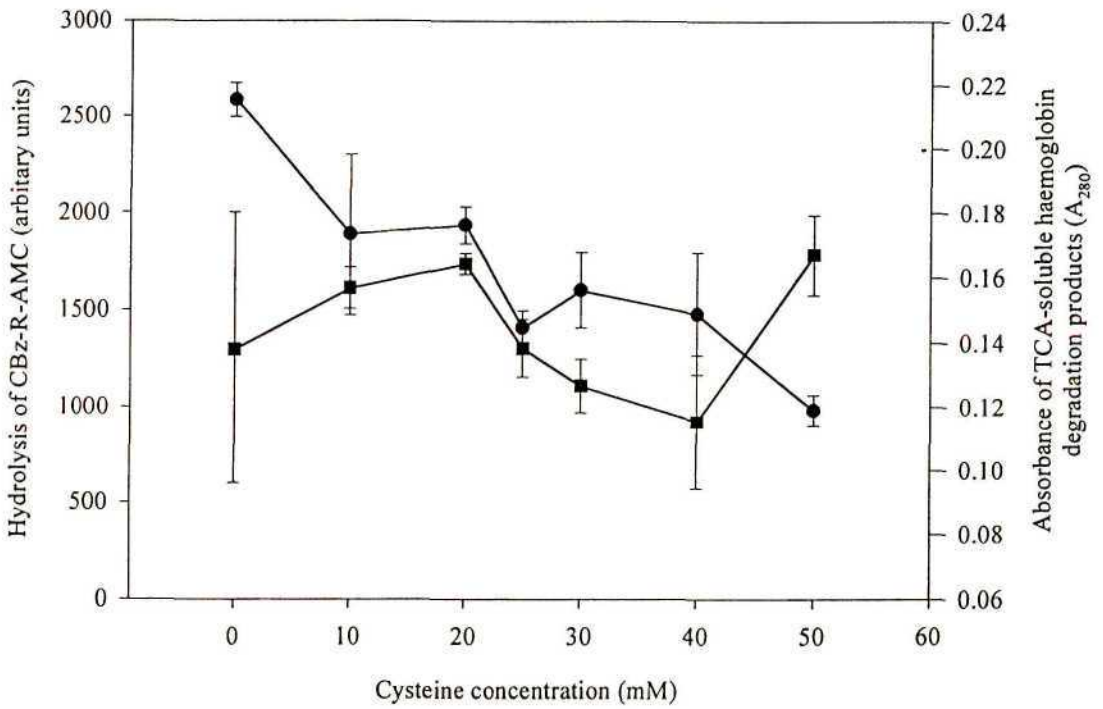

Figure 32. The effect of cysteine on trypsin hydrolysis of CBz-R-AMC and haemoglobin.

Trypsin was incubated in assay buffer containing cysteine concentrations ranging from 1-50 mM (Section 4.10.1). The activity of the enzyme against CBz-R-AMC (●) and haemoglobin (■) was determined under assay conditions described in Section 4.10.2. The data represents the mean ± s.d. (n=3).

Despite the mechanistic advantages of cysteine proteases, serine proteases are much more prevalent in mammalian proteolytic systems. This suggests that the reason that cysteine endoproteases were selected for use in the endosomal system might have more to do with

their ability to be regulated by the redox environment, and/or their stability to highly reducing conditions.

With increasing concentrations of cysteine, the activity of trypsin against CBz-R-AMC progressively decreases (Fig. 32). Trypsin hydrolysis of haemoglobin shows increased activity until 20 mM cysteine, but decreases thereafter. There is an increase in haemoglobin proteolysis at 50 mM cysteine. The cause for this increase in not known. The activation of proteolysis against haemoglobin at relatively low concentrations of cysteine is probably due to the synergistic effects of hydrolysis and reduction on the haemoglobin substrate (Kooistra *et al.*, 1982). These effects are significant enough to offset the denaturation of the enzyme observed with the activity of the enzyme against CBz-R-AMC. However, at higher thiol concentrations (> 20 mM) the denaturation of the trypsin is too complete, resulting in decreased hydrolytic yields.

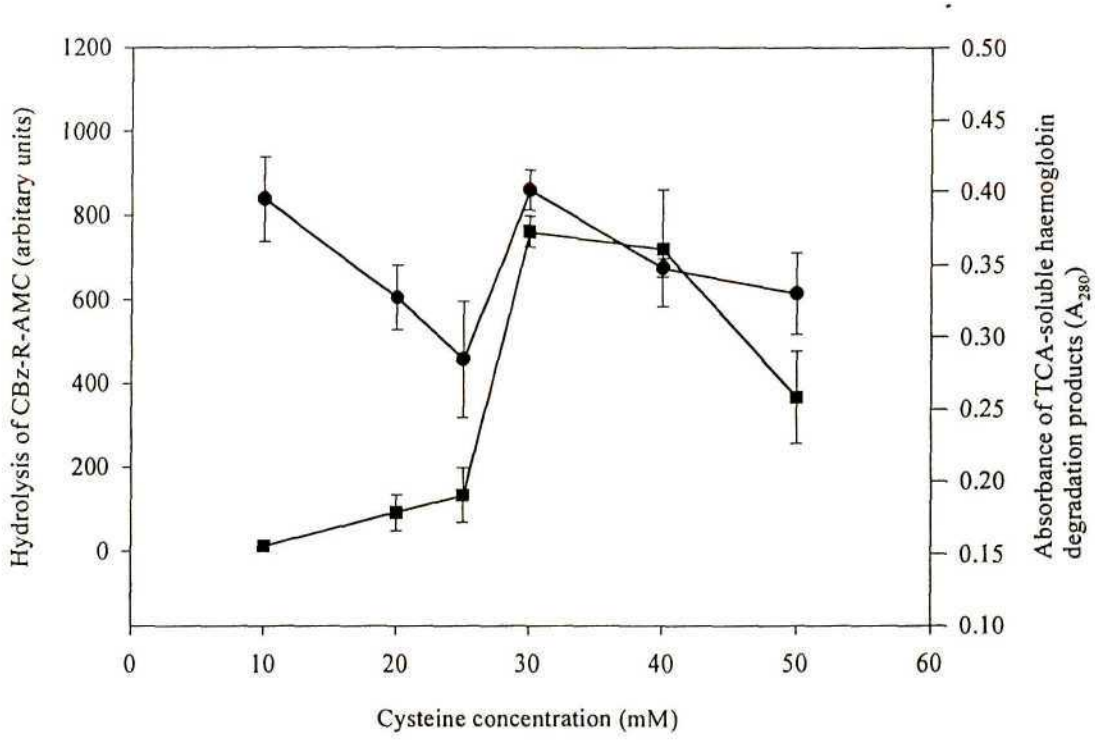

Figure 33. The effect of cysteine on papain hydrolysis of CBz-R-AMC and haemoglobin at pH 7.0.

Papain was incubated in assay buffer containing cysteine concentrations ranging from 1-50 mM (Section 4.10.1). The activity of the enzyme against CBz-R-AMC (●) and haemoglobin (■) was determined under assay conditions described in Section 4.10.2. The data represents the mean ± s.d. (n=3).

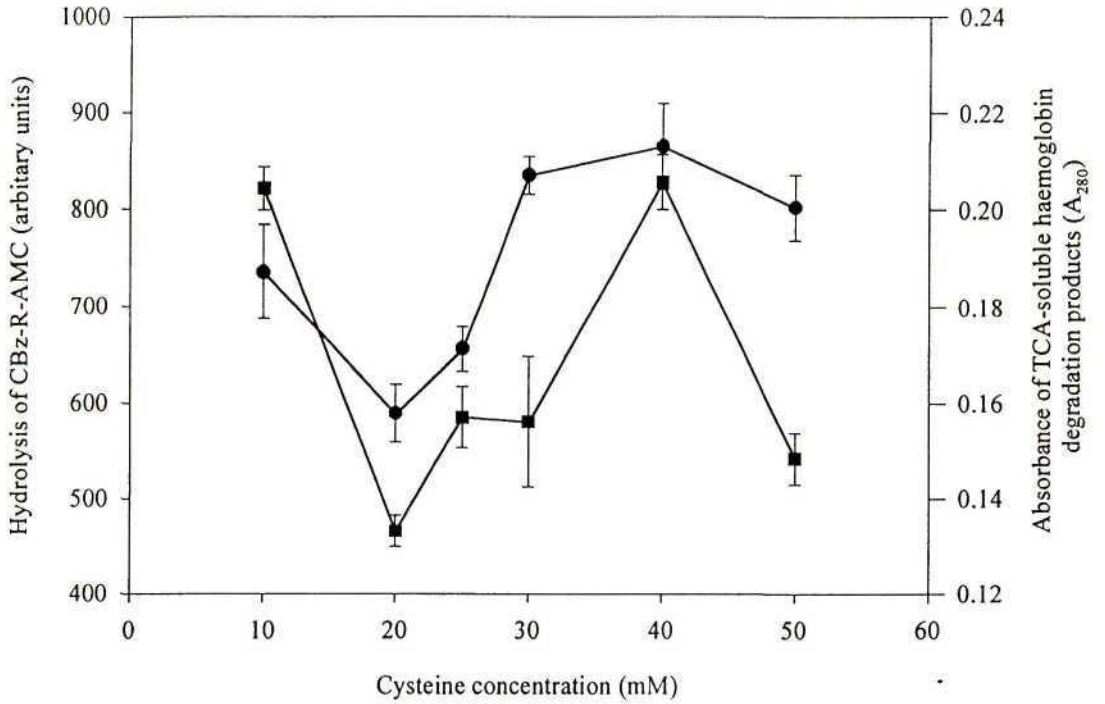

Figure 34. The effect of cysteine on papain hydrolysis of CBz-R-AMC and haemoglobin at pH 6.0.

Papain was incubated in assay buffer containing cysteine concentrations ranging from 1-50 mM (Section 4.10.1). The activity of the enzyme against CBz-R-AMC (●) and haemoglobin (■) was determined under assay conditions described in Section 4.10.2. The data represents the mean ± s.d. (n=3).

The neutral to alkaline pH optima of serine proteases would also preclude their use as lysosomal endoproteases. At these higher pH's reductive denaturation of the enzymes would be more favoured than at lower pH's. In contrast, and whilst cathepsin B does show a pH dependent stability to reducing conditions, the enzyme appears to be stable to high concentrations of thiol (Section 4.5). Serine proteases have evolved by divergent evolution (Rawlings and Barrett, 1994) and the factors discussed above must therefore be tested over a range of serine proteases before any definite conclusions are reached.

At pH 7.0, increases in cysteine concentration, until 25 mM, cause a decrease in papain activity against CBz-R-AMC (Fig. 33). Thereafter, however, papain hydrolysis of the fluorometric substrate appears to be activated by cysteine, with a maximum at 30 mM cysteine. In contrast, hydrolysis of haemoglobin appears to be stimulated by increasing cysteine concentrations, with a maximum at 30 mM cysteine. At pH 6.0, hydrolysis of haemoglobin and of CBz-R-AMC shows a similar response to changes in cysteine concentration, with a local minimum at 20 mM cysteine and a maximum at 40 mM (Fig. 34).

The hydrolysis of CBz-R-AMC proceeds at similar rates at pH 6.0 and pH 7.0. However, against haemoglobin, papain is considerably more active at pH 7.0. The difference between the hydrolysis of the fluorometric and haemoglobin substrates may reflect the effect of pH on the reducing power of a fixed concentration of thiol (Segal, 1976).

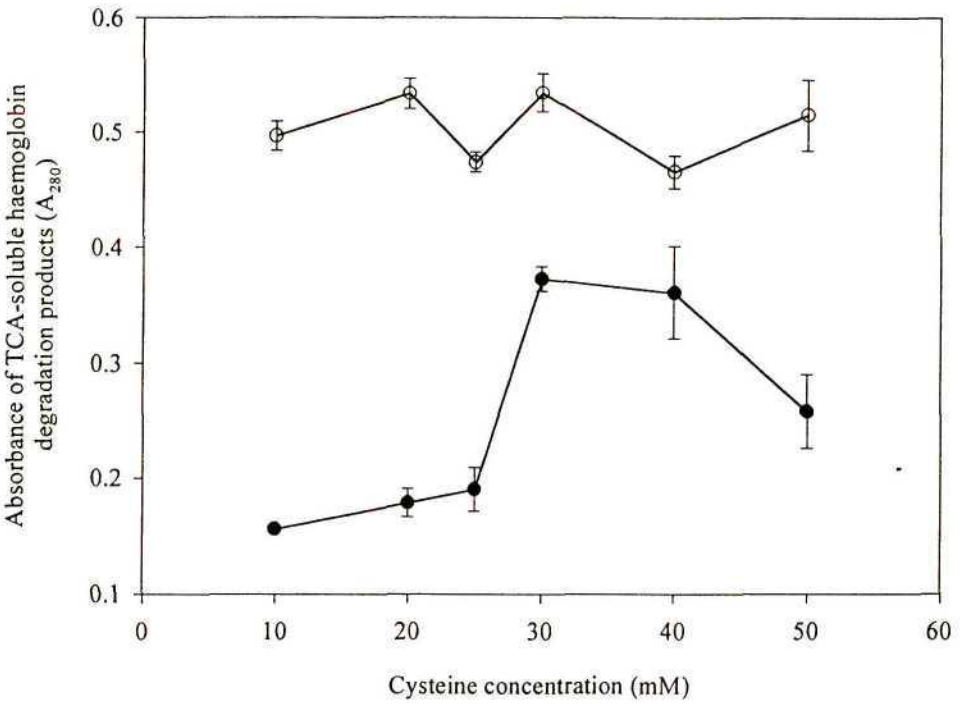

Figure 35. The effect of cysteine:cystine buffers on papain hydrolysis of haemoglobin at pH 7.0.

Papain was incubated in assay buffers containing 1 mM cystine and with cysteine at concentrations ranging from 1-50 mM (O). These reactions were compared to the activity of the enzyme without cystine in the same cysteine concentration range (●). The buffers and assay conditions are described in Sections 4.10.1 and 4.10.2. The data represents the mean ± s.d. (n=3).

Papain hydrolysis of haemoglobin is stimulated by cystine at both pH 6.0 and pH 7.0 (Fig. 35-36). As described for cathepsin B hydrolysis of haemoglobin, this effect is a synergystic effect between and the enzyme and the disulfide, and may be related to the oxidising effect of cystine. At first glance these results appear to be contradictory to those obtained above where increasing the reducing conditions stimulated proteolysis. However, in the envisioned redox system cysteine and cystine would act in concert to stimulate proteolysis: cysteine to activate cysteine proteases and induce the loosening of substrate structure, and cystine to ensure that aggregation of substrate due to hydrophobic interactions is inhibited.

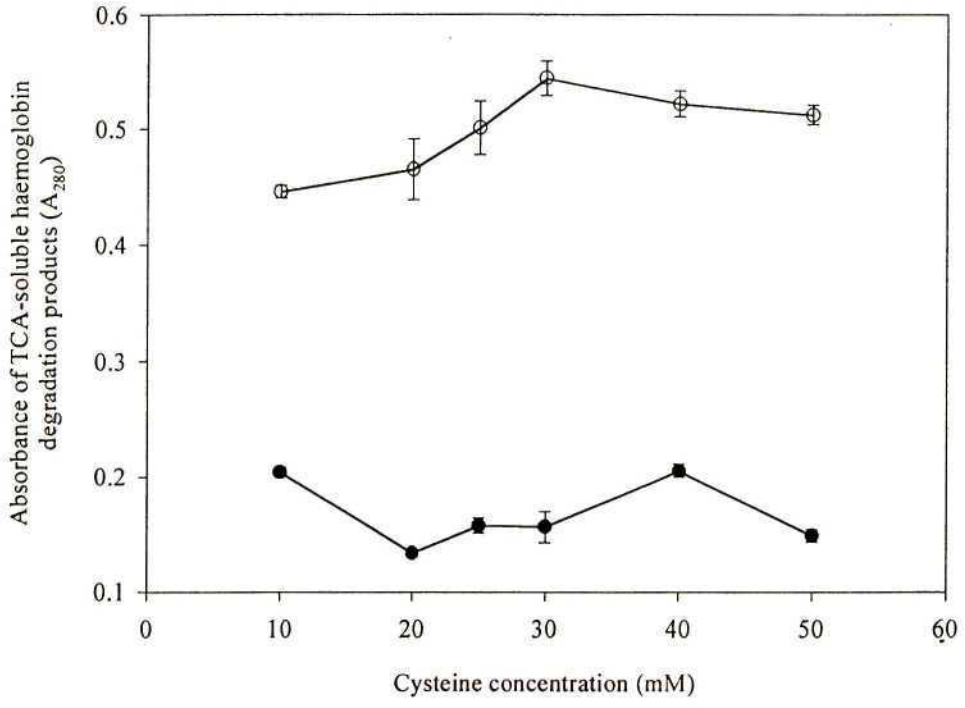

Figure 36. The effect of cysteine:cystine buffers on papain hydrolysis of haemoglobin at pH 6.0.

Papain was incubated in assay buffers containing 1 mM cystine and with cysteine at concentrations ranging from 1-50 mM (○). These reactions were compared to the activity of the enzyme without cystine in the same cysteine concentration range (●). The buffers and assay conditions are described in Sections 4.10.1 and 4.10.2. The data represents the mean ± s.d. (n=3).

CHAPTER 5

GENERAL DISCUSSION

At the outset of this study a model was proposed to explain the differences in functional states between late endosomes and lysosomes. Late endosomes have been shown to be the main site of substrate degradation in the endosomal pathway, despite having only a small fraction of the lysosomal hydrolase pool. Lysosomes were proposed to be storage organelles that, when necessary, injected their lumenal content into the late endosome (Tjelle *et al.*, 1996). It was hypothesised that the late endosome contained a more reducing environment that activated cysteine proteases and stimulated proteolysis of substrate due to loosening of its structure. Lysosomes, on the other hand, were envisioned to have relatively oxidising environments that did not activate cysteine proteases. The vast majority of lysosomal endopeptidases are cysteine proteases, and it is believed that they catalyse the rate-limiting steps in substrate hydrolysis (Bohley and Seglen, 1992). Therefore, shutting them down would effectively limit substrate proteolysis.

In order for the lysosome to maintain a relatively oxidising environment, the cysteine and cystine transporters (Pisoni and Thoene, 1991) would have to be selectively distributed between lysosomes and late endosomes, or else function differentially at the respective pH's of these organelles. It is possible that the oxidising state within the lysosomes could be maintained by the inactivation of the cysteine transporter. The cysteine transporter displays a pH optimum of between 7.0 to 7.5. At acidic pH's (< pH 6.4) the transporter shows a marked decrease in activity. Pisoni *et al.* (1990) suggested that this pH optimum for the transporter may exist on both the lumenal and cytoplasmic sides of the membrane. The oxidising environment of the lysosome may be generated by acidification of the lysosome, which would inactivate the cysteine transporter. Also, the overall redox potential within the system would inevitably become more oxidising at a lower pH. The increased oxidation state within the lysosome may however, be incidental to the regulation of proteolysis within the endosomal system (Section 4.6).

Apart from differences in their lumenal redox environment, this model proposed that the injection of proteases into the late endosome from the lysosome represented a second regulatory mechanism for substrate hydrolysis. Either lysosome-derived vesicles or the lysosomes themselves would have to be made competent for fusion with the late endosome. This process is expected to involve v-SNARE-t-SNARE interactions and the activation of the appropriate Rab-protein machinery. Lysosomal endoproteases, inactivated by oxidation, may become reductively activated by cysteine once they enter the reducing environment within the late endosome. The physiological concentrations of cysteine and cystine, as well as their relative concentrations within different parts of the endosomal system are not known. Part of

the problem with quantifying these factors is that the environment is extremely dynamic. Further, cysteine oxidises extremely rapidly (Mansoor *et al.*, 1992) making physical quantification technically difficult. In an attempt to quantify these redox parameters for the proposed redox regulatory mechanism described above, the lysosomal cysteine protease, cathepsin B, was isolated and its behaviour under various reducing conditions was tested.

The studies on the activation of isolated cathepsin B at different concentrations of cysteine, showed the dependence of the reductive activation of the enzyme on pH. It was found that at higher pH's, a lower concentration of cysteine was required to activate cathepsin B when compared to lower pH's (Section 4.5). Thus (oxidised) cysteine proteases are more easily activated at higher, rather than lower pH's. This activation must be balanced by the stability of the enzymes, which appears to be greater at acidic, rather than neutral or alkaline pH's (Dehrmann *et al.*, 1996). Because conditions are more reducing at a higher than lower pH for a given concentration of thiol (Segal, 1976), the cleavage of substrate disulfide bridges might also be favoured at these higher pH's where cathepsin B is more easily activated.

The relationship between cathepsin B reductive activation and pH is consistent with the inverse relationship between pH and standard reduction potential (Segal, 1976), suggesting that the enzyme might have an optimal redox potential. In order to determine this potential, cysteine:cystine buffers were used to activate cathepsin B. The results for the activation of the enzyme using the fluorometric substrate, CBz-R-R-AMC, did not reveal a specific optimal potential. This may be because the system has too many variables that affect enzyme activity. It is also possible that the enzyme has unique potentials geared to different pH conditions. A new approach to tackle this question would be to try quantify the actual physical or chemical changes (e.g. sulfinic acid to thiolate ion) associated with the enzyme's activation. These results also demonstrated that enzyme may be activated at a purported "threshold" potential, and that this potenial may be relatively oxidisng. This result cast doubt on the proposed redox regulation of proteolysis. Once quantified this "threshold" potential may provide a mechanism by which the enzyme is able to operate extracellularly in conditions like cancer (Yan *et al.*, 1998).

The work with cysteine:cystine redox buffers also opened a new branch of enquiry. Unexpectedly, it was found that cystine-containing buffers promoted the hydrolysis of the whole protein substrate, haemoglobin, by cathepsin B, cathepsin D (an aspartic protease) and papain. This effect was not due to the activation of the actual catalytic mechanisms of the enzymes involved, since it affected both the cysteine proteases and the aspartic protease. Further, this activation effect was not detected using fluorometric substrates, and ligand-binding studies indicated that cystine was not binding to a physical site on cathepsin B. Thus, this effect was most probably directed at the haemoglobin substrate and is possibly due to the ability of cystine to prevent aggregation of protein (Raman *et al.*, 1996).

This is the first report to suggest that cystine, as opposed to cysteine, might play an active role in endosomal proteolysis and that it might be more than a by-product of hydrolysis. This finding is supported by the finding that cystine does get imported into the "lysosome" (Pisoni and Thoene, 1991). It is envisioned that cysteine and cystine set up a redox buffer system that activates cysteine proteases and stimulates proteolysis through the reduction of the disulfide linkages in a substrate. This system must however ensure that substrate is not lost through hydrophobic aggregation, a process that is likely to happen as a protein gets broken down from its tertiary structure to its component amino acids. Cuervo and Dice (1998) have shown that the chaperonin, hsc 73, is selectively imported into the endosomal system by its interaction with the integral membrane protein LAMP-2. Whether hsc 73 acts in concert with the cysteine:cystine redox buffer, to prevent inappropriate aggregation in the complex endosomal milieu, remains to be seen.

The results presented here do not actually demonstrate the presence of this system *in vivo*. However, it must be remembered that the existence of such a system has not been described before, possibly because the concept of promoting protein structure within the degradative endosomal environment appears paradoxical to the functions of the system. The possible loss of protein through aggregation, and the rapidity at which it could occur (Goldberg *et al.*, 1991), has perhaps not been properly appreciated. Secondly, most protocols describing the action of lysosomal cysteine proteases against whole protein substrates are often carried out over several hours and without protection against oxygen (see for example Blair *et al.*, 1993 and Buck *et al.*, 1992). The assays described in this dissertation were all carried out over 10 minutes, which is more in keeping with the half-life of a substrate within the endosomal system (Bohley and Seglen, 1992). With buffers not appropriately deoxygenated, the redox conditions within those experiments carried out over several hours without protection against oxygenation, would be expected to change during the assay. Cysteine oxidises extremely rapidly to form cystine (Mansoor *et al.*, 1992), so the results obtained without protection against oxidation may reflect a cystine-mediated stimulation of proteolysis. This would affect the rate of substrate hydrolysis (Section 4.5), and perhaps the stability of the enzymes themselves (Dehrmann *et al.*, 1996).

As this study progressed, the effects of pH on the reductive activation of cathepsin B opened another avenue of enquiry. The differences in reduction potential between the late endosome and lysosome could probably not, by themselves, account for the regulation of hydrolysis. Firstly, the results obtained in Section 4.6 demonstrate that the cathepsin B ca operate under relatively oxidising conditions. Secondly, it has been shown by Meinesz (1996) that the activity of lysosomal acid α-glucosidase is not regulated by changing thiol conditions. An expectation of any regulatory mechanism would be that it would include all hydrolases within the system. Finally, these proposed differences in the redox potential cannot account for the

different morphologies of the two organelles. Late endosomes appear to be complex, relatively large organelles, whilst lysosomes appear to be small highly dense organelles (Griffiths, 1996).

It was suspected that another regulatory mechanism may exist. Clues to the operation of this possible mechanism came from the work of Kostoulas *et al.* (1997). These authors found that elastase and other enzymes found within the azurophil granules of human neutrophils bound to sulfonated glycosaminoglycans by electrostatic interactions at low pH. It was proposed that this was a storage mechanism for these enzymes. A feature of secretory granules is that they contain condensed cores of aggregated proteins, a morphology similar to that described for lysosomes (Griffiths, 1996). The aggregation process begins in the TGN and is complete once the granules have matured. Using bovine pituitary gland cells, it was demonstrated that the granule content proteins and the lumenal 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+}. Quite remarkably, proteins that were destined for constitutive secretion did not aggregate with the granule content and lumenal membrane proteins, and this property may serve as a segregation mechanism for those proteins that need to be stored and those that are constitutively secreted (Colomer *et al.*, 1996).

The pH-dependent (pH 4.8) aggregation of lysosomal enzymes has been described in Chinese hamster ovary (CHO) cells (Buckmaster *et al.*, 1988). This aggregation was disrupted by NaCl, suggesting that an electrostatic mechanism was responsible for aggregation. Horseradish peroxidase (HRP) that had been chased into the CHO cells failed to aggregate with the lysosomal enzymes. As was the case for the secretory granules described above, aggregation was specific. Jadot *et al.* (1997) were also able to show that aggregation of rat liver lysosomal enzymes occurs at low pH. This aggregation process, which occurred between pH 4.5-5.0, 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. Once again 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.

A pH-dependent aggregation mechanism could operate to withdraw lysosomal hydrolases out of the late endosome, or it could just be a feature of the lysosome itself. An advantage of this complexation mechanism, as opposed to a redox-dependent storage mechanism, is that it appears to involve several different types of lysosomal hydrolases from lipases to proteases

(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 the lysosomal hydrolases within their lumens.

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 mechanism as a means of regulating and storing lysosomal hydrolases has been limited because erroneously low pH optima were assigned to the hydrolases (Dehrmann *et al.* 1995), and the assumption that lysosomes (with their corresponding low pH) represented the organelle where the bulk of substrate hydrolysis occurs. The hypothesis described above does not exclude the possibility that the late endosome may fluctuate between alkaline and acidic pH's, allowing for the activation of many different hydrolases with their individual pH optima (Butor *et al.*, 1995). The lysosome with its low pH may complex the lysosomal hydrolases, effectively storing them in a matrix.

The original model for regulating proteolysis along the endosomal pathway described at the start of this dissertation (Chapter 1), may in fact not exist. Our current model now encompasses two systems responsible for the regulation of proteolysis. The first system regulates the injection of proteases into the late endosome, and presumably their exit from this organelle. Once the hydolases have been correctly sorted, a lysosomal storage mechanism may complex the lysosomal hydrolases to membrane glycoproteins at low pH, storing them in a matrix.

The ultimate result of the present study has thus been a model which explains the observations of Tjelle *et al.* (1996), that late endosomes are the main site of substrate degradation, whilst lysosomes are storage organelles. The function of this model *in vivo* needs to be proved and it also raises several questions, viz.:

- *How does the system regulate the injection of hydrolases into the late endosome?* In the discussion above it was hypothesised that the regulatory mechanism was based on the lysosome or lysosomal vesicles acquiring factors that made them competent for fusion with the late endosome. This implies that there is a sensor(s) that detect the presence of substrate within the late endosome. It is also possible that the fusion of the lysosome to the late endosome may be a cyclic event regulated by a rab-GTP/GDP timer. This question is further complicated by the finding by Claus *et al.* (1998) that the lysosomal population is highly heterogenous, suggesting the possibility of different regulatory mechanisms.

- *Is there any evidence that the lumenal endosomal environment is involved in preventing aggregation?* The results of the present study suggest that cystine stimulates proteolysis, but the mechanism by which this occurs still needs to be proved. If this is in fact due to

the ability of cystine to promote substrate conformation, then this could occasion a paradigm shift in the way that substrate hydrolysis along the endosomal pathway is viewed. Substrate hydrolysis may be far more regulated than previously imagined. Apart from their broad substrate specificity, pH stability, and their efficient catalytic mechanisms, lysosomal cysteine proteases may have been selected for the endosomal system because of their stability in this redox system.

- *Is there a correlation between the pH and density of lysosomes?* The evidence for the use of acid-dependent aggregation as a storage mechanism in secretory granules and neutrophil granules has been well documented. If the aggregation dependent storage mechanism described above actually exists in the endosomal system then there should be a correlation between the morphology and density of lysosomes and their lumenal pH. A further intriguing question is how this aggregation mechanism may be regulated.

These and related questions suggest many possible avenues for future research. Basic research of this nature will undoubtedly provide further insights, which may have significant, though unforeseeable, applications in many areas of applied medicine.

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