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Lysosomal metal, redox and proton cycles influencing the CysHis cathepsin reaction

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In the 1930's pioneers discovered that maximal autolysis in tissue homogenates requires metal chelator, sulfhydryl reducing agent and acid pH. However, metals, reducing equivalents and protons (MR&P) have been overlooked as combined catalytic controls. Three categories of lysosomal machinery drive three distinguishable cycles importing and exporting MR&P. Zn^{2+} preemptively inhibits CysHis catalysis under otherwise optimal protonation and reduction. Protein-bound cell Zn^{2+} concentration is 200–2000 times the non-sequestered inhibitory concentration. Following autophagy, lysosomal proteolysis liberates much inhibitory Zn^{2+}. The vacuolar proton pump is the driving force for Zn^{2+} export, as well as protonation of the peptidolytic mechanism. Other machinery of lysosomal cycles includes proton-driven Zn^{2+} exporters (*e.g.* SLC11A1), Zn^{2+} channels (*e.g.* TRPML-1), lysosomal thiol reductase, *etc.* The CysHis dyad is a *sensor* of the vacuolar environment of MR&P, an *integrator* of these simultaneous variables, and a *catalytic responder*. Rate-determination can shift between autophagic substrate acquisition (swallowing) and substrate degradation (digesting). Zn^{2+} recycling from degraded proteins to new proteins is a fourth cycle that might pace lysosomal function under some conditions. Heritable insufficient or excess functions of CysHis cathepsins are associated with dysfunctional inflammation and immunity/auto-immunity, including diabetic pathogenesis.

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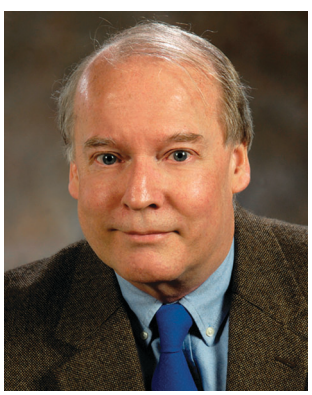

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1. Introduction

1.1. Four lysosomal cycles

Despite differences in substrate preferences and functions, lysosomal CysHis proteases share much in common. In enzyme assay their maximal catalytic rate requires EDTA and DTT under acid-optimal pH. Recent advances reveal a correspondence between this chemical technology and Nature's engineering. Metals, redox and protons (MR&P) are regulated by lysosomal machinery as well as enzymologists. These combined variables can grade CysHis catalysis from completely "off" to maximally "on"[1,2] (Fig. 1). Many lysosomal enzymes cooperate in hydrolysis of macromolecular bonds; however, the triple responsiveness of CysHis cathepsins is unique.

Long ago our group wondered whether MR&P factors might influence CysHis catalysis in lysosomes as well as enzyme assays. We developed a reliable bioassay to measure the release of ^{3}H-leucine from degraded proteins of a perfused tissue. Interventions in redox metabolism or metal homeostasis were affirmative[3,4] (not fully reviewed here). In the following decades, the genome was sequenced; and proteins regulating lysosomal MR&P have been identified. Dysregulations of lysosomal MR&P and CysHis cathepsins are now known to be a causative factor in major human diseases.

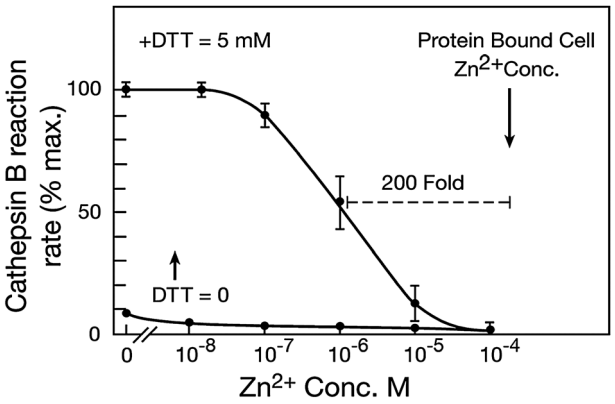

Fig. 1 Zn^{2+} concentration inhibiting cathepsin B under optimal reduction and protonation. Zn^{2+} was removed from purified bovine cathepsin B by EDTA followed by exhaustive dialysis. The protease was preliminarily oxidized to the presumptive sulfoxide by incubation in air-equilibrated water. Reaction rate was assayed as previously described by standard methods using fluorimetric detection of the hydrolysis of carbobenzyloxy-ArgArg-AMC (previously reported in Lockwood 2010). The inactive enzyme was placed in reaction buffer (optimal pH of 5.5) containing the indicated Zn^{2+} concentrations; and substrate was added. A low activity of the oxidized enzyme was observed (bottom trace). DTT reducing agent (5 mM) was then added to the same reactions; and the reaction rate was again measured. These measurements reveal that a low concentration of Zn^{2+} preemptively inhibits cathepsin B in the presence of excess DTT activator and optimal pH. Thus, Zn^{2+} can be an overriding factor limiting the peptidolytic mechanism. Findings were indistinguishable with several L type CysHis cathepsins from various species. Zn^{2+} concentration bound to cell proteins. Gross, protein-bound Zn^{2+} concentration per unit of cell volume was measured in perfused myocardial tissue by standard methods using atomic absorption after complete acid hydrolysis of proteins (Lockwood 2010). Extracellular fluid was preliminarily replaced by Zn^{2+} free perfusion; and results were corrected for extracellular volume. The protein-bound Zn^{2+} concentration/unit cell volume (200 μM) is 200–2000 times the range of Zn^{2+} concentrations that inhibits CysHis proteases (0.1–1.0) μM or less. However, DTT binds added Zn^{2+} and decreases its inhibitory potency (see text). Without DTT, Zn^{2+} partially inhibits at the lowest concentration shown (0.01 μM).

This perspective integrates the catalytic properties of CysHis cathepsins with disparate advances in machinery regulating lysosomal MR&P. Normal functions of CysHis cathepsins are revealed by the consequences of their heritable dysfunction. First, deletion of a single CysHis cathepsin gene can slow lysosomal catalysis.[5] CysHis cathepsins can serve as lysosomal ''pacemakers'' under some conditions. Second, single and multiple CysHis cathepsin gene deletions protect against progressive auto-immune tissue damage,[6,7] and inflammatory damage from pro-catabolic cytokines.[8] Inflammation and auto-immunity are ''double trouble'' underlying the pathogenesis of several diseases, including diabetic progression.[9] Third, heritable dysregulations of lysosomal MR&P in certain cell types are associated with diseases of insufficient or excessive lysosomal function (see below).

Metallomics and redox proteomics have become routine concepts.[10–13] However, ''multiomics'' has not yet entered the lexicon. Thus far, no unifying redox code or metal code has been deciphered in prokaryotes or the compartmentalized cells of eukaryotes. Primordial ''omics'' has been complicated by the evolution of higher-order protein structure, and separately regulated compartments. There is one type of catalytic mechanism, in one type of compartment, which retains a primitive code of omic control. A proton-responsive enzyme with cross-membership in the redox proteome and metal-interactive proteome is controlled by several simultaneous variables. Semanticists have not yet characterized pH-dependent proteins as the ''protonosome''. However, the lysosomal compartment regulates these three variables; and these variables regulate CysHis catalysis. Cys confers redox-responsiveness, and His adds metal-responsiveness; together they confer proton-responsiveness. It is proposed here that the CysHis dyad is (a) a sensor of the vacuolar environment of metals, redox and protons, (b) an integrator of these simultaneous variables, and a (c) catalytic responder to cell regulation of the three categories of factors.

Understanding the control of CysHis cathepsins requires decoding the combined effects of metals, redox and protons on the peptidolytic mechanism. In the absence of metals the activity is maximal under optimal pH near 5.5, and reductive activation by several mM DTT (Fig. 1). Any of the three members of the governing triumvirate can limit CysHis peptidolysis. However, Zn^{2+} (0.1–1.0 μM) is a preemptive inhibitor despite maximal stimulation by protonation and reduction. The disulfhydryls of DTT bind Zn^{2+}; therefore the actual inhibitory Zn^{2+} concentration in Fig. 1 is much less than the added concentration. Prior to addition of DTT some amount of inhibition can be observed at the lowest Zn^{2+} concentration shown (0.01 μM) (Fig. 1, bottom trace). The Zn^{2+} sensitivities of several B- and L-type cathepsins were similar. The triple responsiveness of CysHis cathepsins corresponds to the three categories of lysosomal import–export cycles (Fig. 2). These inputs and outputs displace vacuolar MR&P from equilibrium conditions. CysHis cathepsins are ''wired'' to the currents of metals, reducing equivalents and protons created by these cycles. A fourth lysosomal cycle transfers Zn^{2+} from degraded protein binding sites to new proteins.

1.2. Scope and limitations of the abstract theory

Prior to consideration of the present theory, several misperceptions must be dispelled. It is generally presumed that autophagy is the rate-determining step in lysosomal function. However, autophagy and intra-vacuolar degradation can be compared to swallowing and digesting. If intra-vacuolar hydrolysis were always faster than substrate acquisition, then lysosomes would never become loaded with un-degraded macromolecules. In most images of the cell, some lysosomes appear loaded. Micro- and macro-autophagy have been reviewed many times in recent years, (but not here!). In contrast, intra-vacuolar catalysis has evaded attention thus far. The rate-limitation on lysosomal function can shift between substrate acquisition and substrate degradation under various conditions.

In the absence of inhibitory metal concentration CysHis peptidolysis can function outside the lysosome or cell at a submaximal rate until the enzyme becomes oxidized[14] (illustrated by the bottom trace in Fig. 1 under low metal concentration). Diverse lysosomal functions have evolved in

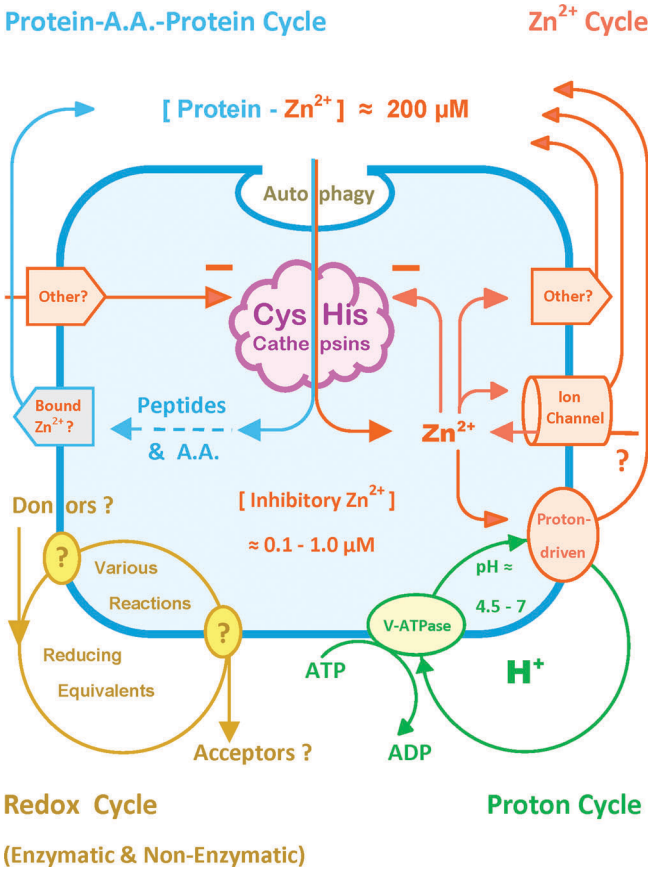

Fig. 2 Recognition of four lysosomal cycles for future correlation with CysHis cathepsin activity. The peptidolytic chemistry of Fig. 1 challenges molecular biology to account for lysosomal metal, redox and proton (MR&P) cycles that determine the catalytic environment of CysHis cathepsins. This simplified composite is intended to accommodate future advances in the machinery involved and its integrated control. Following substrate uptake, intra-vacuolar proteolysis liberates a large amount of Zn^{2+} from macromolecular binding sites; other inhibitory metals might be relevant (see text). Without export, lysosomal Zn^{2+} would rapidly accumulate to an inhibitory concentration (Fig. 1); and CysHis catalysis would be self-limited. The recycling of released metals from degraded proteins to new proteins might be a rate-determining factor in recycling amino acids into new proteins. The V-ATPase is a major driving force for the export of Zn^{2+} and other metals *via* the proton-driven Slc11A1 and other transporters. Conceivable importers and exporters labeled "other" include ATP-binding cassette (ABC) transporters which transport a wide variety of substances that bind Zn^{2+}, *e.g.* peptides, glutathione, organic cations *etc.* (and see text). Some TRP channels passively conduct Zn^{2+} down its electrochemical gradient inward as well as outward. Some TRP channels are phosphorylated or pH dependent; and some rectify conductance. Multiple, enzymatic and non-enzymatic redox reactions might influence CysHis cathepsins. Pathway(s) transferring reducing equivalents into and out of the lysosome are presently unknown. This abbreviated summary excludes such relevant topics as the cystine exporter, cystinosin, GSH/GSSG transport, lysosomal dipeptidase *etc.* (see text).

relation to the diversity of specialized cells that employ them. CysHis cathepsins have extra-cellular functions, *e.g.* degradation of the extra-cellular matrix. This review is generalized to the essential CysHis reaction mechanism within vacuoles. Several topics are postponed until better understanding of their relevance to lysosomal cycles, *e.g.* the integrity of the lysosomal membrane,[15] activation of the "inflammasome" cascade,[16] endogenous protease inhibitors,[17] metabolic and post-receptor signals regulating the lysosomal machinery, *etc.*

The prokaryotic CysHis catalytic mechanism evolved into cathepsins, caspases and calpains. MR&P control of intra-vacuolar catalysis does not conflict with present understanding of extra-lysosomal CysHis proteases (see Section 7).

Lysosomal electrophysiology is presently controversial;[18–21] and, understanding of integrated Zn^{2+} transport is presently at the beginning stages. Acceptance of this incomplete theory of catalytic control does not require knowledge of the lysosomal membrane potential or control of all ions. Indeed, this theory provides a guide to unanswered questions.

When disparate advances from many investigators are assembled, Nature's logic becomes self-evident. The present theory is difficult to distinguish from a fact; however, lysosomal MR&P cycles are initially described here with broad simplicity. Introduction at the preliminary stage is justified by the immediate relevance to pathology and therapeutics.[2]

2. A lysosomal cycle importing and exporting protons

Names for different structures of the lysosomal system include: dense granules, pre-lysosomes, endosomes, phagosomes, pinocytic vesicles, macro-autophagic and micro-autophagic vacuoles, endoplasmic reticulum-lysosome fusion bodies, lysosomal-mitochondrial fusion bodies ("lysondrion"), expired lysosomes, residual bodies, exosomes and even "regurgisomes". These various structures have a wide range of pH, redox properties, metal contents and catalytic activities. Here, the criterion of a functional lysosome is a catalytically active vesicle hydrolyzing peptide bonds.

2.1. Import of lysosomal protons

Enzymatic and non-enzymatic peptide bond hydrolysis ("splitting by addition of water") is highly dependent upon proton concentration. However, lysosomal proton transport has acquired additional significance in light of other processes that depend on it (Fig. 2). Inactive lysosomes can exist with neutral pH; and active vesicles can decrease pH below 5. Progress in regulation of the vacuolar proton pump has been slow due to the complexity of this machine. The structure and function of the V-ATPase in relation to other ions has been reviewed in detail.[21]

If the proton pump is completely inactive, the non-sequestered vacuolar Zn^{2+} concentration at pH of 7 might be similar to the cytoplasm. When the proton pump is active, Zn^{2+} export lowers the inhibitory metal to the permissive concentration for CysHis cathepsin activity (Fig. 1 and 2). The non-sequestered, vacuolar Zn^{2+} concentration presumably varies inversely with the proton gradient that drives export. However, Zn^{2+} and proton concentrations might not exist in direct inverse proportionality. Increasing proton concentration drives Zn^{2+} export while simultaneously protonating the CysHis reaction mechanism, and releasing large amounts of Zn^{2+} from degraded binding sites. Various TRP

channels can be influenced or controlled by Zn^{2+}, Mg^{2+}, pH, phosphorylation and others.[22,23]

2.2. Export of lysosomal protons

A significant route of proton export is direct coupling to metal export (see below). The ability of lysosomes to acidify implies that proton export and leak are small relative to import under maximal function of the V-ATPase.

3. A lysosomal cycle importing and exporting reducing equivalents, and reducing and oxidizing CysHis cathepsins

The chemistry of protein thiolation–dethiolation corresponds to the pharmacology of Kosower's sulfhydryl oxidizing agent (diamide) *vs.* Cleland's sulfhydryl reducing agent (dithiothreitol). Diamide accepts reducing equivalents from two sulfhydryls in a two-step oxidation catalyzing the formation of disulfides.[24,25] DTT undergoes internal oxidations of its disulfhydryls to disulfides as it transfers to acceptors. Thiolation–dethiolation was demonstrated using one dimensional gel electrophoresis of tissues exposed to diamide.[26,27] An increase in glutathione disulfide causes corresponding increase in protein-mixed disulfides. An idealized theory suggests that the GSSG/GSH redox ratio controls the functions of some proteins. Redox buffering remains valid in purified reactions; however, application to the compartmentalized cell under non-equilibrium conditions requires modification.[25,28]

Evidence for lysosomal machinery associated with thiolation–dethiolation can be summarized with a few findings. In 1993 Stephen *et al.*[29] reported that an inactive papain disulfide can be reductively reactivated by NADPH, thioredoxin reductase and thioredoxin in a reconstituted reaction. It was simultaneously reported that pharmacologic thiolation–dethiolation caused by diamide and DTT resulted in large changes in protein degradation in perfused tissue.[3,4] A lysosomal thioredoxin was then described,[30] and found to activate lysosomal proteases.[31] Cathepsin B is responsive to the redox ratio of GSSG/GSH[32] as well as a variety of other oxidants. Assembly of these findings suggests transfer of extra-lysosomal reductive energy to oxidized vacuolar CysHis cathepsins. The exact intermediaries and pathways transferring extra-lysosomal reducing energy into the vesicle are unknown. The ''redox cycle'' shown in Fig. 2 is a generalized composite representing multiple intermediaries and pathways in various cell types and species.

It is now known that thioredoxins and glutaredoxins comprise the major pathways to protein reduction in various compartments. However, thioredoxins and glutaredoxins exhibit cross talk, and some redundant functions.[33] Indeed, thioredoxins of some compartments are glutathionylated. Glutaredoxins and thioredoxins can both reduce GSSG and PrSSG as well as other oxidation products. Oxidized and reduced glutathione are found in lysosomes as well as the endoplasmic reticulum (ER). Lysosomal transport of some form(s) of glutathione is suspected. Thus far, functional glutathione reductase and glutaredoxins have not been reported within lysosomes.

3.1. The oxidation of CysHis cathepsins and export of lysosomal reducing equivalents

Only the reduced sulfhydryl of CysHis cathepsins is catalytically active. Submaximal activity results from partition of the population of enzyme molecules among the reduced active state and all oxidized inactive states. The partition of catalytic cysteine is the net result of all opposing enzymatic and non-enzymatic redox reactions. Catalytic sulfur can exist in a shifting partition among reversible states until it is irreversibly oxidized. The catalytic partner can oxidize to 2-oxo-histidine.[34,35] Extensive histidine oxidation seems likely under extreme conditions in some species. However, the significance of histidine redox in mammalian CysHis peptidolysis represents an important gap in present knowledge.

The best single answer to the question of vacuolar CysHis redox is that there is no single answer. CysHis redox within the lysosome can be compared to a busy intersection with cross-traffic from many transferring pathways. The simplified redox cycle of (Fig. 2) represents a multitude of non-enzymatic and enzymatic redox interactions with proteases. This problem is comparable to other cell compartments.[36] The chemical and biochemical contents of the lysosomal system are undefined to state the least. Lysosomal contents can include any exogenous particle or solute that can be taken up by fluid pinocytosis, phagocytosis or receptor-mediated endocytosis. Moreover, the catabolic vacuole can contain the entire content of the mitochondrion or endoplasmic reticulum. Ongoing processes in mitochondria and ER do not cease instantaneously upon fusion with lysosomes.

Much of what is now known about protein sulfur redox was originally learned using a CysHis protease as experimental tool. Papain permits determination of the redox state of a single protein sulfur site using protease activity as an indicator. This botanical protease has long been available in crystallized form, thereby permitting defined reactions without impurities.[37] Papain can be ''gently'' oxidized under conditions that do not cause irreversible inactivation. Far more is known about papain chemistry in purified reactions than papain biology in the cell. Oxidized derivatives of papain sulfur include sulfenic, sulfinic and sulfonic acid (PrSO, $PrSO_2$, $PrSO_3$),[38] glutathionylations (PrSSG),[39,40] nitrosylations (PrSNO).[41–48] Some ''deep'' oxidation states are not reversible under biological conditions, *e.g.* $PrSO_3$.

Reactive oxygen species (ROS), reactive nitrogen species (RNS) and the redox ratio of GSSG/GSH are known to be cell signals. Protein sulfoxygenations, nitrosylations and glutathionylations control the functions of some proteins. However, defined redox reactions in purified reconstituted systems can differ greatly from *in vivo* reactions. An experimental sulfhydryl oxidant can first oxidize and deplete protective GSH, and then oxidize the unprotected protease cysteine directly. In purified enzyme solutions, hydrogen peroxide produces protein sulfenic acids; and nitric oxide donors produce nitrosylations. When competing GSH

is present, these oxidants first produce GSSG, and then increasingly oxidize proteins as GSH is depleted.[47–49] Any agent that oxidizes GSH to GSSG can secondarily oxidize Protein-SH to Protein-SSG. Therefore, diverse oxidants act initially to increase the GSSG/GSH ratio and secondarily to oxidize protein sulfhydryls.

Pathways transferring reducing equivalents from lysosomal sulfhydryls to oxygen, or other acceptors, are uncharacterized. Work to be done is illustrated by comparison of known features of redox transfer in the endoplasmic reticulum with corresponding unknowns of the lysosome. The ER has an enzymatic relay system transferring reducing equivalents from internal protein sulfhydryls to oxygen in the process of forming internal disulfide bonds of the folded protein.[50–53] The ER has multiple pathways of sulfhydryl oxidation in addition to the ER oxidoreductin system (ERO). Dehydroascorbic acid/ascorbic acid redox has been implicated in ER-associated degradation as well as lysosomal degradation.[36,53] In contrast to cytoplasm, the ER compartment has a high content of glutathionylated proteins and GSSG. Thus far, there has been no suggestion that the ERO relay system is operative in the lysosome after compartmental fusions. Redox implications of lysosomal fusions with other compartments are virtually unstudied.

Speciated metals could participate in the pathways transferring from reduced sulfhydryls of proteins or glutathione to oxygen and other acceptors. (The oxidation state of Zn^{2+} does not change under biological conditions; although some binding sites are redox-responsive.) Speciated metals are mutually reactive with protein sulfhydryls and reactive oxygen species or diatomic oxygen. Fe^{3+}/Fe^{2+} or Cu^{2+}/Cu^{1+} can act as cycling intermediates, and accelerate the transfer of electrons from CysHis cathepsins to oxygen or other acceptors. Speciated metals accelerate the oxidation of CysHis cathepsins in air-equilibrated water. In addition, Fe^{3+} and Cu^{2+} (oxidized) directly inhibit CysHis cathepsins.[1] In contrast, Fe^{2+} and Cu^{1+} (reduced) are not potent inhibitors of CysHis cathepsins. Under extreme oxidative conditions (or reductive deficiency) in lower species, ferric and cupric states almost certainly attain sufficient concentrations to directly inhibit CysHis cathepsins.

ROS and RNS are suspected of an influence on CysHis cathepsin function in some specialized cell types.[38,41,43–48] The superoxide radical is reported to have a role in controlling CysHis cathepsins in macrophages and dendritic cells.[54–57] Net superoxide levels result from the relative rates of production by NADPH oxidase *vs.* elimination by superoxide dismutase as well as non-enzymatic reactions. The complicated reactions derived from the superoxide radical can produce an oxidative signal in low amounts and pathogenic oxidations in higher amounts. ROS or RNS might also interact with some forms of Fe or Cu to oxidize CysHis cathepsins. GSH and the anti-oxidant enzymes, superoxide dismutase, catalase, and peroxidase might all be involved in lysosomal redox of various specialized cell types.[58] Mitophagy transfers the entire content of the mitochondrion to the catabolic fusion vacuole with unknown consequences.

3.2. The import of lysosomal reducing equivalents and reduction of CysHis cathepsins

In the absence of glutaredoxins, GSH activates CysHis cathepsins with less potency than equimolar DTT.[32,49] Several mM GSH alone can cause an appreciable activation of cathepsins in reaction without metals. Cathepsin B activity is responsive to the ratio of GSSG/GSH as expected of a typical ''redox-buffered'' protein *i.e.* increasing GSSG causes increasing inhibition.[32] Cathepsin L from various species exhibits less sensitivity to the redox ratio of GSSG/GSH (unpublished observation). It should be cautioned that GSSG peptide might compete with peptide substrates without forming a true disulfide bond.

In viable tissue, the responsiveness of proteolysis to diamide *vs.* DTT is consistent with a contribution of thiolation–dethiolation to the control of proteases.[3,4] In perfused myocardial tissue non-toxic diamide concentrations (100 μM or less), reversibly inhibited all of lysosomal and some of extra-lysosomal proteolysis (^{3}H-leucine release from proteins). Following discontinuation of short diamide exposures, cell reductive metabolism could reverse the inhibitory action. After prolonged diamide exposures of 1–2 hours inhibitions were not completely reversible. Following oxidation, GSSG is rapidly exported from this tissue.[59] Exogenous GSH is not rapidly taken up. Therefore, perfused tissue was exposed to supra-physiologic extracellular GSH concentration in order to replete cell GSH following diamide exposure. Despite toxicity of 1 mM GSH, this high concentration reversed the anti-proteolytic effect of diamide beginning after a lag period.

Consistent with studies on perfused tissue it was reported that reducing equivalents from NADPH can be transferred to the reduction of oxidized papain disulfide *via* thioredoxin reductase and thioredoxin.[29] A lysosomal thioredoxin was later identified by its mannose-phosphate destination tag.[30] The lysosomal reductase co-localizes with vacuolar proteases.[31] This reductase reduces internal disulfide bonds and unfolds antigens in specialized cells of the immune system.[30,31,60] However, the lysosomal reductase has been identified in other cell types. Thioredoxins exhibit broad substrate specificity. A reductase that can reduce internal disulfide bonds of proteins can also reduce oxidized sulfur at a protease surface. The lysosomal reductase can activate pro-CysHis cathepsin B and D[31] (and see Section 6). Genetic polymorphism in the expression of the lysosomal thiol reductase is associated with diabetic pathogenesis.[61]

The extra-lysosomal origins of vacuolar reducing equivalents, and pathways into the vesicle are unknown (represented as ''various reactions'' in Fig. 2). The source of reduction of extra-lysosomal thioredoxins is NADPH *via* thioredoxin reductase. Reduction of the intra-lysosomal thioredoxin-type reductase is puzzling insofar as NADPH dos not cross membranes. Multiple enzymatic and non-enzymatic factors might serve as redox shuttles from extra-lysosomal compartments into the lysosome.[32,62] Much about redox-dependent proteolysis might be learned from the unusual metabolism of apicomplexan (malarial) parasites[63] and other parasites.

Many active transporters are capable of moving a wide variety of endogenous and xenobiotic molecules with molecular weights of several hundred *e.g.* the ABC transporters (see below). The lysosome is believed to import and export various peptides with broad specificity[64,65] (Fig. 2). Cystinosis

is a lysosomal storage disease causing accumulation of vacuolar cystine and lysosomal insufficiency. Cystinosis involves heritable dysfunction of the lysosomal cystine exporter ''cystinosin''. Cystinosis reveals the normal function of this transporter (not shown in Fig. 2). Lysosomal export of cystine is apparently necessary because the thiol reductase cannot reduce this disulfide bond or cannot reduce it fast enough. Cystine/cysteine is part of redox signaling.[66] However, the possible roles of cystine/cysteine and GSSG/GSH in lysosomal redox have not been characterized. The lysosome might import and export many redox-active or metal-binding substances that are yet to be discovered.

4. Lysosomal cycles importing and exporting Zn^{2+} and other metals

A slight elevation of extra-cellular Zn^{2+} can decrease lysosomal proteolysis[47]. Therefore, lysosomal Zn^{2+} regulation somehow communicates with extra-lysosomal and extra-cellular Zn^{2+}.[67] Lysosomal Zn^{2+} regulation involves trans-membrane Zn^{2+} gradients; therefore, it is responsive to Zn^{2+} in other cell compartments. In addition, cytoplasmic Zn^{2+} might influence signal networks and autophagy.

A vast literature on metal regulation is complicated by several factors. Compartmental Zn^{2+} regulation results from an interaction among multiple transport mechanisms with opposing vectorial directions. Active lysosomes function under non-equilibrium conditions, with an import/export current of metal ions (Fig. 2). Reported driving forces for lysosomal Zn^{2+} transport include a proton gradient, a bicarbonate gradient,[68] the Zn^{2+} electrochemical gradient, ATP and perhaps others. Different metals share some of the many transport mechanisms summarized below. For example, Zn^{2+} interacts competitively with the regulation of Fe^{2+}.[69–72] Although Zn^{2+} is emphasized here, Cu^{2+}, Fe^{3+} and some other metals can also inhibit CysHis cathepsins.

It is difficult to determine the subcellular location and function of a transporter or channel. The position and transport direction of membrane-associated protein can change under membrane fusions.[73] The identical metal transporter might be found in the plasma membrane-early endosome of one cell type, and the lysosome of another. Indeed endosomes can recycle to the cell surface or fuse with lysosomes. The locations and functions of various metal transporters, and the primary metal(s) transported might differ in various specialized cells.

The electrophysiology integrating lysosomal Zn^{2+} translocation with other lysosomal ion regulation is virtually unstudied. This topic involves the lysosomal membrane potential and regulation of all other ions, *e.g.* bicarbonate, chloride, magnesium, calcium, *etc.*[74] The present perspective defers to references[18–23,75,76] for discussion of lysosomal electrophysiology. If Zn^{2+} binding to macromolecules or peptides is tight, and dissociation is slow, transport does not conform to principles of ionic electro-physiology. In addition, metallothioneins have a redox-dependent Zn^{2+} release mechanism.[77,78]

Recent progress in Zn^{2+} biology has been explosive. Confirmed and likely lysosomal transporters are described by several names due to the history of independent discoveries. The Transporter Classification Database (TCDB) used here is an organization approved by the International Union of Biochemistry and Molecular Biology (IUBMB).[79] Many metal translocators might be directly or indirectly relevant to the lysosome. Accordingly, Fig. 2 accommodates anticipated progress by representing lysosomal Zn^{2+} regulation with known and unknown driving mechanisms.

As a beginning, lysosomal Zn^{2+} homeostasis must be organized as (a) the regulation of the ''mobile'' or non-bound metal ion, and (b) the regulation of the bound metal that is co-transported with other substances. Cell Zn^{2+} regulation includes compartmental sequestration, high affinity protein binding, and low affinity buffering involving many substances *e.g.* citrate, phosphate, amino acids *etc.*[80–83] Terms used to describe the biologically effective Zn^{2+} concentration include ''free'', ''active'', ''non-bound'', ''un-buffered'', ''non-sequestered'', ''mobile'', ''chelatable'' *etc.* Perfectly free biological Zn^{2+} is believed to be in the picoMolar range. For ionic Zn^{2+} the relevant consideration is the effective electrochemical Zn^{2+} gradient across the lysosomal membrane, and not the total Zn^{2+} concentration. The cytoplasmic activity of Zn^{2+} is mimicked by addition of approximately 1–3 μM Zn^{2+} to a mixture of endogenous Zn^{2+} buffers at pH 7. In catalytically active lysosomes the non-bound Zn^{2+} concentration is presumably maintained near the permissive concentration of 0.1 μM (Fig. 1). In inactive vesicles at neutral pH the Zn^{2+} concentration might be much higher without the driving force of the proton gradient.

Zn^{2+} has six coordination sites. Binding can decrease effective collisions and ''potency'' of Zn^{2+} without eliminating all reactivity of sites remaining exposed. Therefore, the proper consideration for enzyme inhibition is the effective concentration of interactive Zn^{2+} sites remaining exposed under all endogenous ligands at some particular pH. Indeed, for some enzymes the effective inhibitory potency of some Zn^{2+} complexes can be greater than the free ion.[84] Coordination of Zn^{2+} with some binding sites need not eliminate fluorophore interactions of ligands remaining exposed. Imaging with cytofluorimetric Zn^{2+} indicators does not distinguish the sequestered and non-sequestered lysosomal Zn^{2+} concentration, or electrochemical gradient.[85–88]

Several types of Zn^{2+} translocators have been identified; however, their driving forces, controls and functions are not well understood. Transporters of non-bound Zn^{2+} include several families. The mammalian genome codes a family of 14 Zip transporters (TCDB:Slc39a) and 9 ZnT transporters (TCDB:slc30a).[82] Slc39a14 (or Zip 14) has been reported to be a metal/bicarbonate symporter.[89] Slc30a (ZnT) transporters function as Zn^{2+}/H^{+} exchangers. Slc39a transporters are reportedly found in the plasma membrane, whereas Slc30a are have been found in various organelles. The two divalent metal ion transporters (Slc11A1 and Slc11A2) couple the uphill transport of Zn^{2+} and downhill transport of protons. These proteins also transport Fe^{2+} in some cell types. ATP binding cassette

(ABC) transporters couple metal transport to ATP hydrolysis. ABC transporters are fundamental to metal regulation in bacteria;[90,91] however, this mechanism is not well characterized in eukaryotes. Humans have more than 50 ABC transporters. Mammalian ABC transporters are known to transport a wide variety of substances that bind Zn^{2+}(see below). In addition to transporters, mammals have 8 passive Transient Receptor Potential (TRP) channels. These trans-membrane channels conduct non-sequestered metal ions down their electrochemical gradients. Calcium channels have been reported to pass Zn^{2+}.[92,93] Omitting the ABC transporters and calcium channels, the combined number of transporters and channels involved in ionic Zn^{2+} regulation exceeds 30. Many of these might influence lysosomal regulation directly or indirectly. At present, the locations, vectorial orientations, and interactions among these many transporters are not known.

Lysosomal import and export of Zn^{2+} that is bound to other substances can differ markedly from transport of ionic Zn^{2+}.[94] Approximately 10% of cell proteins and many other substances can bind Zn^{2+} with a wide range of affinities.[95] It is suggested that Zn^{2+} might exchange between high-affinity protein binding sites directly without dissociation into solvent.[96] The stable Zn^{2+} interactions with 2Cys2His of many Zn^{2+} finger proteins are well studied;[97] however, Zn^{2+} interactions with 1Cys1His sites of proteases have received little attention. Regulatory Zn^{2+} structures with Cys near His include an ion channel;[98] and metallothionein[99] as well as the familiar Zn^{2+} finger motifs.

The ABC transporters include diverse members with a broad range of specificities. Peptides are exported from lysosomes and also imported into lysosomes, probably by ABC transporters.[64,65] GSH and GSSG form protective complexes with metals including Zn^{2+}; and these might share Zn^{2+} transport *via* ABC transporters.[100–102] ABC transporters are involved in defenses against metal toxicity in eukaryotic cells.[103,104] Much about lysosomal metal regulation might be learned from defense against overload of biological and non-biological metals. The broadly specific, ATP-dependent, organic cation transporters might transport Zn^{2+} that is bound to other substances. Finally, bound Zn^{2+} might accompany the translocation of peptides *via* endocytosis, exocytosis and organelle fusions *e.g.* mitophagy or autophagy. Indeed, a fraction of most proteins can be found in lysosomes, presumably with any Zn^{2+} that is bound.

4.1. Selected topics in the lysosomal import of Zn^{2+} and other metals

It can be readily estimated that a major route of lysosomal Zn^{2+} acquisition in active lysosomes is autophagic uptake of protein-bound Zn^{2+} followed by release upon proteolysis. Approximately 10% of the proteome has Zn^{2+} binding sites; and many more proteins bind with lower affinity. Metallothioneins exhibit differential protease susceptibility and intra-lysosomal metal release.[105] The gross, protein-bound Zn^{2+} concentration is 200 μM (Fig. 2). Basal protein turnover of a typical cell is approximately 2–3% h^{-1}. Therefore, each hour 2–3% of 200 μM protein-bound Zn^{2+} is released from binding sites. This release rate would increase non-sequestered cell Zn^{2+} concentration by 4–6 μM per hour if it were uniformly distributed. Approximately half of total protein degradation occurs within lysosomes in most cell types. The active lysosomal volume is only 10–15% of total cell volume. Therefore, 2–3 μM Zn^{2+} is liberated from degraded proteins into 10–15% of cell volume each hour. This liberated Zn^{2+} concentration would be 200–2000 times the concentration that inhibits CysHis cathepsins (0.1–1.0 μM, Fig. 1) if released from binding sites without vacuolar export. Without Zn^{2+} buffering by 5 mM DTT, some inhibition was observed at 0.01 μM Zn^{2+} (Fig. 1, bottom trace). The 0.01 μM inhibitory Zn^{2+} concentration is approximately 10 000 fold below the protein-bound cell Zn^{2+} concentration of 200 μM. The lifetimes of lysosomes in various cells are not known with precision. However, if vesicles persist for only 0.5–1.0 h, then the Zn^{2+} liberated from degraded proteins would rapidly accumulate to an inhibitory concentration in the absence of export. Possible inaccuracies of these estimates are quite conservative. Thus, active lysosomes function under a cycle of massive Zn^{2+} acquisition on substrate proteins, liberation by proteolysis, and export by multiple routes (Fig. 2).

Non-lysosomal compartments might import ionic Zn^{2+}, and then fuse with lysosomes secondarily. Indeed, yeast cells have a Zn^{2+} storage vacuole for future growth under Zn^{2+} deficiency.[106] Some mammalian cells sequester Zn^{2+} in vacuoles.[107]

4.2. Selected topics in the lysosomal export of Zn^{2+} and other metals

TRPM1–TRPM8 comprise a group of eight ion channels which can pass metals in either direction down the electrochemical gradient.[108] Zn^{2+} is reported to regulate its own conductance through a TRP channel with allosteric regulatory kinetics.[109–115] Zn^{2+} conductance through some TRP channels is believed to be partially rectified;[110,111] although the present relevance is unknown. Some TRP channels are responsive to pH and phosphorylation.[22,23,109–114] The TRPML-1 channel is of great interest to lysosomal function.[114–117]

The TRPML-1 is found in the lysosomal membranes of diverse cell types. A telling feature is that heritable deficiency of TRPML-1 channel function results in a lysosomal storage disease known as mucolipidosis-IV.[117] Many variants of lysosomal storage diseases affect all cell types; however, the life-limiting injury is to neural tissue. Mucolipidosis results in accumulation of undegraded macromolecules in large lysosomes of the brain. Eichelsdoerfer *et al.*[118] reported: ...“the loss of TRPML1 function results in intracellular chelatable zinc dyshomeostasis. We propose that chelatable zinc accumulation in large lysosomes and membranous vacuoles may contribute to the pathogenesis of the disease and progressive cell degeneration in ML-IV patients”... This disease of denatured protein accumulation implies that a normal function of the TRPML-1 is net export of lysosomal Zn^{2+} and perhaps other cell Zn^{2+} regulation. Mitochondria are also affected. The drosophila ortholog of TRPML-1 is necessary for normal lysosomal function.[119] The TRPML-1 does not account for all routes of lysosomal Zn^{2+} export. Complete loss of lysosomal Zn^{2+} export would presumably be

embryolethal. Loss of TRPML-1 channel function is also reported to cause disruption of lysosomes and release of cathepsin B into the cytoplasm.[81,120] The normal TRPML-1 channel somehow serves in ionic and volume control of the vacuole.[121–124] The TRPML-1 channel convincingly reveals that Zn^{2+} export is related to lysosomal function as well as the CysHis cathepsin reaction mechanism (Fig. 1 and 2).

The macrophage protein named ''Natural Resistance Associated Membrane Protein 1 (NRAMP1 or SLC11A1) is also fundamentally related to lysosomal function. This protein was originally discovered by the association of microbial infections with loss of its function, hence the name. This gene family has two members: Slc11A1 and Slc11A2; the former is of most interest here. Slc11A1 is expressed in phagocytes, and exports metals from the phagosome. The driving force for uphill lysosomal metal transport is coupling to downhill proton transport. Curiously, insufficient function of Slc11A1 is somehow associated with inability to defend against microbes; and excessive function is associated with a variety of hyper-immune immune syndromes independent of microbes.

Heritable or regulatory alteration of protein function can be caused by structural gene mutations, variations in gene copy number, altered regulation of gene transcription, alternative transcript splicing, RNA interference *etc.* Beyond the present scope, these combined mechanisms can lead to a wide range of protein content and function among individuals. Regardless of causative mechanism(s), the present theory is the only way to account for all pathogenic results of both insufficient and excess functions of lysosomal metal export.

Slc11A1 can export multiple divalent metal cations from lysosomes, including Zn^{2+} and Fe^{2+}. The normal function of Slc11A1 was believed to be starvation of phagocytosed microbes for Fe^{2+} as a growth requirement in specialized phagocytes. This microbicidal mechanism is currently known as ''metal withdrawal defense''.[125] The mechanism of immune deficiency that is associated with decreased Slc11A1 function is currently believed to be a failure to starve phagocytosed microbes for Fe^{2+} and other metals. It has not been appreciated that metal withdrawal defense might interact with an additional microbicidal mechanism of the Slc11A1 exporter. Normal export of inhibitory metals from phagosomes/lysosomes could activate CysHis cathepsins, and increase the degradation of captured microbes while starving them for metal requirements. Conversely, deficiency in the lysosomal export of inhibitory metals would explain protection of endocytosed microbes against destruction by CysHis cathepsins.

Whereas deficient Slc11A1 function is associated with immune deficiency, heritable up-regulation is associated with hyper-immunity. The hyper-immunity that is associated with excessive expression of Slc11A1 is obviously not related to starvation of phagocytosed microbes for Fe. Therefore, the theory of ''metal withdrawal defense'' cannot account for the pathogenic results of excessive function of the Slc11A1 transporter. It is well known that lysosomal CysHis cathepsins are required for proteolytic antigen processing to immunogenic peptides and presentation to specialized cells of the immune system.[126–128] Most investigators believe that excessive cell content of the Slc11A1 somehow increases lysosomal proteolytic processing of auto-antigens to immunogenic peptides, thereby increasing auto-immunity. Several CysHis cathepsins are involved in antigen processing.[7] Autoimmunity can be associated with heritable up-regulation of Slc11A1 expression. Many studies demonstrate a relationship between excessive expression of the Slc11A1 transporter and the auto-immunity of diabetic progression.[129–142] The result of excessive function of Slc11A1 confirms the relevance of metal export to lysosomal proteolytic function.

Hyper-immunity might be also be associated with excessive lysosomal export of inhibitory Zn^{2+} and Fe^{2+} *via* Slc39A8 (Zip-8). Slc 39A8/ZIP-8 is localized to the lysosomes of at least some cell types. In other cells Slc 39A8 is located on the cell surface and in early endosomes.[69–72] In a model of T-cell activation and antigen processing, lysosomal Slc 39A8 is increased in association with interferon production.[143] A role of Slc39A8 in lysosomal Zn^{2+} export upon T-cell activation has been suggested. Such an interpretation would be consistent with an associated role of Slc39A8 in cathepsin activation.

In summary, the normal functions of lysosomal metal regulation in catabolic control are revealed by the results of dysregulations. Defective TRP channel structure, or down-regulation of the proton-driven exporter alone can decrease lysosomal Zn^{2+} export, and cause immune deficiency or a lysosomal storage disease. At the other extreme, excessive function of proton-driven, lysosomal, metal export is associated with excessive lysosomal metal export, and diverse hyper-immune syndromes, *e.g.* progressive diabetic tissue damage. Optimal lysosomal function exists within some intermediate range between pathogenic excess and insufficiency. All of these observations can be explained by a regulatory contribution of Zn^{2+}, and other metals, to CysHis cathepsin function.

5. The energy dependence of lysosomal cycles

The hydrolytic reaction catalyzed by CysHis cathepsins is driven by energy released from the peptide bond. Therefore, the catalyzed reaction is ultimately exergonic. However, maintenance of the maximally active CysHis catalyst has a continual energy requirement. ATP is required for metal export and protonation of the CysHis reaction; and reducing energy is required to oppose protease oxidations. The high-energy phosphate network and redox network are distinguishable systems. After a century of investigation, relationships between the high-energy phosphate network and the redox network involve unanswered questions.[144,145] Nonetheless, interventions with traditional agents of known actions provide limited information for correlation with future advances.

Experimental demonstration of an ATP requirement for a hypothetical process can lead to the conclusion that fluctuations in cell ATP content control that process. However, a requirement for ATP does not necessarily demonstrate regulation of the process by the permissible range of cell ATP fluctuations.

Many observed phenomena are co-variables of injury and death under extreme experimental ATP depletion. If the ATP content of a tissue can be decreased to the minimal amount required for sustained viability without changing lysosomal function, then the ATP requirement for proton pumping would not seem to control lysosomal cycles. In perfused myocardium, repeated mitochondrial poisoning and ATP depletion did not decrease lysosomal proteolysis until immediately before functional demise and death.[4] Thus, the minimal amount of ATP needed to sustain viability of myocardial tissue also suffices to sustain lysosomal cycles. In marked contrast, interventions with non-toxic concentrations of sulfhydryl oxidants and reductants cause large fluctuations in bulk protein degradation with absolutely no change in tissue ATP content or contractile function. Unnecessarily high or prolonged exposures to redox active agents were injurious. Conclusions from mammalian myocardial tissue might not typify the wide range of metabolic conditions observed in all plants, animals and microbes. It is likely that severe, sublethal ATP depletion can limit lysosomal proton pumping and catalysis in some species under some conditions.

6. Beyond the fact that pro-cathepsin B is a member of the Zn^{2+} – binding ''metallome''

Most of the cell content of lysosomal hydrolases consists of inactive pro-enzymes. The simultaneous activation of more than 60 acid hydrolases by cleavage of pro-regions remains unexplained.[14] Active lysosomal proteases can cleave the pro-regions of inactive pro-proteases and other pro-hydrolases. Therefore, all lysosomal proteases can be considered to be convertases activating other lysosomal enzymes. For this reason, it has been presumed that the activation of pro-hydrolases is determined by the ongoing rate of vacuolar proteolysis; and this is not inconsistent with the present theory.

The control of ongoing CysHis peptidolysis by MR&P cycles could influence normal or abnormal activation of other pro-lysosomal enzymes. Some investigators suspect that lowering pH alone causes increasing activation of pro-hydrolases.[21] However, lysosomal pH is only one component of the MR&P triumvirate. It has been suggested that the lysosomal reductase system can contribute to activation of pro-cathepsins B and D.[31] However, it has not been suggested that all three lysosomal cycles conspire to determine the activation of all pro-lysosomal enzymes. The present theory agrees that reducing energy and acidity are positive influences on pro-protease activation; however, it has not been considered that Zn^{2+} might preemptively oppose these activating influences.

Pro-cathepsin B was discovered objectively in a screen of the Zn^{2+} – binding proteome.[10] A speculative possibility is that cathepsin B is a primary ''trigger protease'' for the activation of other lysosomal enzymes, at least in cell types where it is abundant. Cathepsin B can be promptly activated *ex vivo* either by non-specific inter-molecular proteolytic cleavage of the pro-region or decreasing pH below 5. Cathepsin B has a unexplained structure called the occluding loop. The pro-region of Cathepsin B is folded over its catalytic dyad. It has been suggested that cathepsin B auto-activates by self-cleavage of its own pro-region under a pH-dependent conformational change in the occluding loop.[146] Bound Zn^{2+} at the catalytic dyad might suppress such self-cleavage of the pro-region by enzymatic activity.

It remains to be determined whether cathepsin B is the primary convertase activating other proteases in response to lysosomal cycles. Metallomic techniques might not detect metal binding to other pro-proteases that are present in lower amounts. Curiously, gene deletion reveals that cathepsin B is not critical to the life of the mouse due to a degree of functional redundancy. Therefore, cathepsin B cannot be the only converting mechanism activating lysosomal enzymes. The question of whether pro-hydrolase activation by MR&P involves cathepsin B or all CysHis cathepsins might depend upon the relative enzyme contents of particular cell types. The liver has a very high content of cathepsin B. Massive hepatic failure causes the lethal, life-limiting tissue damage 12–24 h following overdose of TNFα. As described above, knockout of the cathepsin B gene alone protects against this lethal receptor-mediated liver damage.[8]

7. The recycling of amino acids from old proteins to new proteins in association with recycling of metals from degraded binding sites to new binding sites

Recycling of Zn^{2+} from degraded proteins to new proteins has all features expected of a crude coordinator of lysosomal and perhaps some extra-lysosomal pathways of proteolysis. Limited studies indicate that extra-lysosomal proteolysis is indeed responsive to redox and Zn^{2+}. This responsiveness includes caspases,[147] calpains,[148] and the ubiquitin-proteasome pathway.[149–153] Cathepsin B has been found in extra-lysosomal compartments, and is also suspect of extra-lysosomal functions. Oxidative stress is a widely used term that has not been defined. Moderate oxidative stress (or reductive insufficiency) can be a non-injurious part of metabolic control. Moderate oxidative conditions can decrease apoptosis in some cell types.[154] However, changes induced by Zn^{2+} and/or redox can involve higher order enzyme structure as well as the catalytic mechanisms discussed here.[149,150]

The reaction mechanism of an active caspase or calpain might serve its specialized function at a small fraction of the maximal catalytic rate. Indeed, non-injurious operation of CysHis cathepsins *in vivo* might correspond to a small fraction of the optimized rate shown in Fig. 1. Relative sensitivities of lysosomal and extra-lysosomal proteolytic pathways to Zn^{2+} and redox are uncharacterized. Zn^{2+} might contribute to coordination of multiple proteolytic pathways. Interestingly, metalloproteases and metallopeptidases require Zn^{2+} for activity, and do not require a reductive activator. Lysosomal dipeptidase is such a metal-redox independent enzyme.[155] In summary, diverse extra-lysosomal proteolytic systems are sensitive to Zn^{2+}

and redox; however, this responsiveness includes higher-order protein structure.

Pharmacologic interventions against excessive CysHis cathepsin activity can have effective anti-inflammatory actions against diabetic pathogenesis. The most effective drug against progressive diabetic pathogenesis is an anti-lysosomal "zincophore".[2] However, drug development[156,157] must be cautious. Normal lysosomal function requires a mid-range between excessive and insufficient function. Many other causes of denatured protein accumulation might interact with excessive anti-lysosomal therapy *e.g.* Parkinson's disease,[158] Alzheimers disease,[159] and many lysosomal storage diseases.[160]

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