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Rapid Letter

Cathepsin B Responsiveness to Glutathione and Lipoic Acid Redox

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ABSTRACT

Some subcomponents of cell protein degradation exhibit an unexplained reductive energy requirement; and diverse cysteine proteases are among multiple effector mechanisms requiring reduction. Present studies investigated whether cathepsin B activity is graded in response to (a) reduced glutathione (GSH) and dihydrolipoic acid (DHLA) concentrations, (b) their redox ratios, and (c) their differential potencies and efficacies. Purified bovine cathepsin B activity was assayed with carbobenzyloxy-Arg-Arg-aminomethylcoumarin by standard methods following inactivation by spontaneous air oxidation. Endogenous GSH concentration (2–3 m*M*) maintained 30–40% of the maximal cathepsin B reaction rate observed under dithiothreitol (5 m*M*). Following activation with GSH, the cathepsin B reaction rate was inhibited in proportion to nonphysiologic GSH:GSSG redox ratio above 1% oxidized (*e.g.,* 85% inhibited at 3 m*M*:2 m*M*). Thus, cathepsin B can be redox buffered by the GSH:GSSG ratio. DHLA was identified as a potent cathepsin activator with threshold near 1 μ*M* and 80% maximal activation near 10 μ*M*. Conversely, oxidized lipoamide disulfide inhibited cathepsin B over 5–250 μ*M*. DHLA at 5–50 μ*M* superimposed severalfold additional activation upon the stable submaximal cathepsin B reaction rate maintained by endogenous GSH concentration (2–3 m*M*). Cell protein degradation was bioassayed by release of [^{3}H] leucine from the biosynthetically labeled rat heart under nonrecirculating perfusion. The pro-oxidant, diamide (100 μ*M*), reversibly inhibited 80% of basal proteolysis. Supraphysiologic extracellular DHLA (80 μ*M*) doubled the basal rate of averaged cell protein degradation in 15 min. Thus, the cell redox system buffers an intermediate rate of protein degradation, which can be decreased by supraphysiologic exposure to diamide pro-oxidant or increased by DHLA reductant. *Antioxid. Redox Signal.* 4, 681–691.

INTRODUCTION

SEVERAL SUBCOMPONENTS OF CELL PROTEIN DEGRADATION require reductive energy; however, some proteolysis is redox-independent (25–27). A remarkable diversity of redox-responsive proteolytic effector mechanisms is now well established (20, 25, 33, 40).

Following introduction of the effective reductant, dithiothreitol (DTT), 40 years ago, many redox-responsive protein functions have been characterized under experimental conditions of nearly maximal reduction. However, protein function can be graded in response to (a) total concentration of reductant, *e.g.,* reduced glutathione (GSH), (b) the reduced to oxidized ratio of redox couples, *e.g.,* GSH:GSSG, and (c) the differing potencies and maximal efficacies of redox-active agents, *e.g.,* GSH versus DTT (11, 27). Proteins can be enzymatically reduced by multiple pathways, including thioredoxins, glutaredoxins, and direct, NADPH-dependent reductases (2, 11, 27, 30). In addition, a large diversity of nonenzymatic factors can also influence redox-responsive protein functions. The multiplicity, diversity, and redundancy of protein redox mechanisms complicate attempts to compare isolated assay conditions with intracellular enzyme function.

Much of redox-responsive cell protein degradation can be inhibited by nonspecific, redox-inactive, cysteine protease active site ligands (25); however, the intracellular redox

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control of various cysteine proteases is uncertain. Most cells contain at least 20 cysteine proteases, including cathepsins, calpains, and caspases. Despite the diversity of their individual functions and controls, cysteine proteases share the namesake cysteine catalytic amino acid. This active-site catalytic mechanism oxidizes spontaneously and requires reductant for maximal assayed activity (5, 37, 38). Following pro-region cleavage, cysteine protease reaction rates are generally considered to be either "on or off" with little consideration given to the possibility of graded activity in response to the surrounding redox environment. Oxidative inactivation of cysteine proteases can presumably override redox-independent controls, and might be independently operative. It is conceivable that redox buffers, activators, or inhibitors could be among multiple intracellular rate-limiting factors influencing proteases under some conditions.

Dihydrolipoic acid (DHLA) is the only permeant endogenous disulfhydryl that can be used to impose experimental reductive stress within the hierarchy of intracellular redox potentials (9, 12, 26, 29, 32, 39, 40). Following synthesis from octanoic acid within mitochondria, DHLA is enzymatically ligated to several reductases as prosthetic group (12, 26, 32, 39). A metabolic role of nonligated DHLA has not yet been determined. The intramolecular oxidation of DHLA to the cyclic dithiolane ring exhibits a large negative redox potential (18, 19). Free DHLA, DHLA-ligated enzymes, and intact mitochondria can reduce a large variety of metabolites, including dehydroascorbic acid and GSSG (19, 23, 40). DHLA can also reduce thioredoxins, and this pathway has been speculatively suggested in protein reduction (10).

The present report indicates that a model cysteine protease conforms to general features of enzymes that are buffered at a submaximal rate by the GSH:GSSG redox status. Second, DHLA is a surprisingly potent cysteine protease activator that can superimpose severalfold additional activation upon the submaximal cathepsin B reaction rate maintained by endogenous GSH status. Third, using radiolabeled perfused tissue as bioassay, large decreases or increases can be superimposed upon the basal rate of intracellular protein degradation by oxidative action of diamide or reductive action of DHLA, respectively.

EXPERIMENTAL PROCEDURES

Protease assay

Using standard methods, mature, fully active bovine spleen cathepsin B (Sigma) was assayed fluorometrically with *N*-carbobenzyloxy-Arg-Arg-7-amino-4-methylcoumarin (Z-Arg-Arg-AMC) (25 μ*M*) (Bachem) in acetate buffer (50 m*M*), pH 5.5. Major findings were similar over a wide range of Z-Arg-Arg-AMC concentrations of at least 10–70 μ*M* or using Z-Phe-Arg-AMC (25–40 μ*M*). Results are indicative of saturated proteases. None of the present assays were influenced by quenching or addition of fluorescence. Reaction rates were statistically fitted over a time period of steady-state reaction progress using the DeltaSoft reaction kinetics program ($R > 0.95$). Activators or inhibitors were added to ongoing reactions in 200-fold concentrated water or dimethyl sulfoxide (DMSO) solutions causing 0.5% volume dilution. DMSO solvent controls were assuredly negative as also described by others. Slight irregularities upon some additions are transient optical artifacts associated with vigorous mixing.

Perfusion of hearts

Hearts from Sprague–Dawley rats were perfused as nonloaded Langendorff preparations by methods previously described (24). A constant flow of 7.0 ml/min sustained a low perfusion pressure of 40 mm Hg and rigorous contractile function for 5 h. The nonrecirculating perfusate (95% O_2/5% CO_2) routinely contained Krebs–Henseleit salts, glucose (10 m*M*), and physiological concentrations of citrate, pyruvate, lactate, complete amino acids, and bovine serum albumin (0.2%), all adjusted to pH 7.42 after addition. pH was monitored continuously.

Measurement of protein degradation in myocardium

Proteins were biosynthetically labeled by infusion of L-4,5-[^{3}H]leucine (40–60 Ci/mmol, 4.5 μCi/ml) for 10 min in the absence of nonradioactive perfusate leucine. Nonradioactive leucine (0.75 m*M*) was subsequently added to prevent reincorporation of label. Results are nearly identical when physiologic leucine concentration (0.1 m*M*) is substituted for supraphysiologic leucine; however, experimental changes in protein synthesis can cause a slight change in postcursor externalization due to reincorporation. Results are qualitatively identical when sufficient amounts of [^{3}H]leucine are injected into the rat 4 h prior to perfusion. The nonrecirculated effluent perfusate was collected at 2-min intervals in a fraction collector. Trichloroacetic acid soluble radioactivity was determined. A two-component equation describing the progress of macromolecular [^{3}H]leucine remaining in myocardial protein over 5 h has been previously described (25). In the present studies, the rapidly degraded subcomponent of protein degradation was eliminated during a preliminary 3 h degradative period prior to the observation period. Under constant intracellular conditions, the rate of total [^{3}H]leucine release from 3–5 h postlabeling declines continually in proportion to the progressively declining amount of undegraded labeled proteins remaining. Control values of protein degradation are presently illustrated as the normalized 100% value of the declining baseline of [^{3}H]leucine release. From 3–5 h postlabeling, each preparation can be accurately used as its own experimental control by extrapolation of the statistically fitted baseline of [^{3}H]leucine release over the following 1.5-h period of degradative inhibition, as previously described (24).

The present sustained effect of DHLA on increased [^{3}H]leucine release is not due to a transient release of intermediate cytoplasmic amino acid pools, but rather a true elevation in protein degradation. The cytoplasmic [^{3}H]leucine pool is exchanged with a half-time of ~0.5 min, as compared with the much longer averaged half-time of protein degradation. Artificial elevation in [^{3}H]leucine release exclusively from the intermediate cytoplasmic compartment cannot be sustained for more than 2–3 min in this nonrecirculating preparation. Muscle can metabolize leucine; however, the percent per minute metabolism was determined to be a very

small fraction of the rapidly exchanging intracellular leucine pool (for review, see 25).

Drugs were infused into flowing perfusate in 500–1,000 concentrated solutions immediately above the heart. DHLA was infused in a DMSO solvent at a final concentration of 0.01%. It was rigorously determined that DMSO solvent alone caused no change in myocardial protein degradation at 0.05% or 0.5%, *i.e.,* less than the experimental uncertainty of ±1–2%.

Reagents

DL-DHLA and lipoamide disulfide were from Sigma–Aldrich. DHLA is supplied sealed under argon gas due to its instability under oxygen (22, 23). Upon breaking the seal, DHLA was quickly dissolved in DMSO at 100 m*M* and tightly capped until prompt use. GSH and DTT were freshly dissolved at the time of use. Other materials were as previously described (24).

RESULTS

DHLA is a prompt potent reductive activator of cysteine proteases

In addition to metal ions, several oxidative reactions can reversibly alter the function of protein sulfhydryls *ex vivo* (5, 9, 20–22, 27, 35, 38). Reversible air oxidation of protein sulfhydryls is generally attributed to sulfoxide formation (5). Addition of cathepsin B to stirred water (4°C) equilibrated with atmospheric oxygen caused inactivation reaching ~97–99% in 5–10 h. Inactive cathepsin B in water (4°C, pH 7) was stable for at least 12 h, and could be stored frozen for at least several weeks. Cathepsin B inactivated by this method could be promptly reactivated by 5 m*M* DTT within several minutes.

After such oxidative inactivation in water, the percentage of active cathepsin B remaining ranges near 1% of recoverable activity. The results of Fig. 1 in arbitrary units were selected so as to illustrate the time course of separate measurements shown in Fig. 2 as percent maximal recoverable activity. In Fig. 1, the slope of the nearly inactivated protease is arbitrarily defined as 1 so as to illustrate a 99% inactivated reaction rate. Upon addition of 2 μ*M* DHLA, the reaction rate in Fig. 1 increased promptly from 1to 58, corresponding to slightly more than 50% reversal of a 99% inactivation.

The actual potency of DHLA action almost certainly deviates from that experimentally observed. Using fresh DHLA preparations, several percent of maximal protease reactivation was detected at 1 μ*M*. However, despite capped storage, the minimally detectable protease activation increased to ~20 μ*M* DHLA in ~1 day due to spontaneous oxidation (see Experimental Procedures). Second, in fresh DHLA preparations, an optimal peak activation occurred near 1 m*M* DHLA, and higher concentrations were less effective. In experiments such as illustrated in Fig. 2, the exact contour and maximal value of the curve near the peak activation varied in relation to the age of the DHLA preparation due to an inhibitory action of the oxidized form (discussed below). Third, maximal possible DHLA action required vigorous mixing with fluid

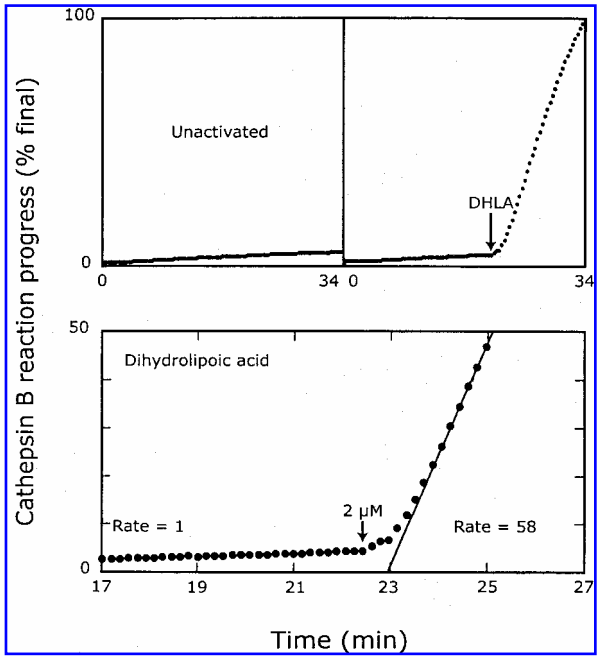

FIG. 1. Activation of cathepsin B with DHLA. Prior to the time shown, cathepsin B was spontaneously inactivated by placement in air-equilibrated water. At zero time, cathepsin B was combined with buffer and substrate, revealing a low amount of activity defined arbitrarily as a unit slope of 1 (top left panel). At the time indicated by the arrow, DHLA (2 μ*M*) was added to the separate reaction (top right panel). An area of the top right panel is shown enlarged in the bottom panel. Results are representative of five determinations.

turbulence, suggesting that solubility and diffusion can limit its effect at threshold concentrations. Fourth, binding to the wall of the reaction vessel might also influence the apparent threshold DHLA concentration. Finally, artificial enzyme denaturation by internal structural disulfide reduction can occur at prolonged exposure to high 10–15 m*M* DTT, although the present relevance to 1 m*M* DHLA is unknown. Protease activation can be approximated at an apparent experimental threshold near 1 μ*M* DHLA. A range of 20-80% of maximal protease activation occurred at 5–10 μ*M* DHLA as illustrated from two representative experiments in Fig. 2 (continuous and dashed traces).

Steady-state cathepsin B reaction rate is responsive to GSH concentration alone, and also GSH:GSSG redox status

When added to previously inactivated cathepsin B, GSH (2 m*M*) caused a submaximal reactivation reaching steady state in several minutes (Figs. 2–5). Protease activation was concentration-dependent over the range of 1–10 m*M* GSH, and continued to increase at 10 m*M* GSH (Fig. 2). At the 2–3 m*M* GSH concentration found in most cell types, protease activation was ~20–30% of the reaction rate caused by 2 m*M* DTT in parallel reactions (Figs. 2 and 3).

Under conditions of metabolic sufficiency, glutathione pools are actively maintained 99-99.5% reduced, corresponding to a

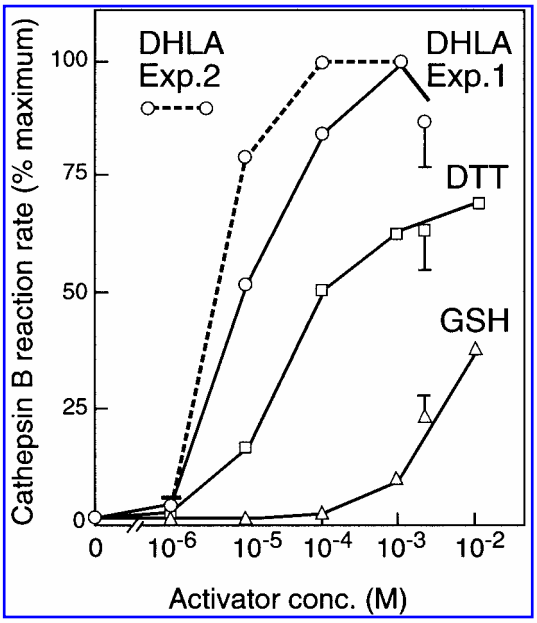

FIG. 2. Concentration dependence of cathepsin B activation with DHLA, DTT, and GSH. In experiment 1, means ± SD are shown at 1 μ*M* and 2 m*M* for each agent. Results with low DHLA concentrations were far more variable than those with DTT or GSH (see text). Shown by the dashed trace are values from a separate experiment (experiment 2) illustrating the upper range of observed DHLA action.

redox ratio of 100:1 to 200:1 (9, 11, 27). At a 99.5% reduced GSH pool of 3 m*M*, the mammalian GSH:GSSG redox ratio would be approximately 3 m*M*:15 μ*M*. Following activation by 3 m*M* GSH, cathepsin B was unresponsive to addition of the basal endogenous 15–30 μ*M* GSSG concentration (Fig. 3A and data not shown). In cathepsin B reactions activated over the range of 2–5 m*M* GSH, it was rigorously determined that addition of 1% oxidized GSSG did not cause inhibition exceeding the several percent experimental precision (data not shown). Therefore, under typical mammalian intracellular conditions of 99% reduced GSH:GSSG, only the reduced GSH influences the reaction rate appreciably. However, in the presence of 3 m*M* GSH, addition of GSSG concentrations above 1% inhibited cathepsin B with a steep but broad concentration dependence extending to ~85% inhibition at a grossly supraphysiologic ratio of 3 m*M* GSH:2 m*M* GSSG (Fig. 3A). The likely upper mammalian metabolic range of 100–200 μ*M* GSSG caused ~10–20% inhibition of the cathepsin B activity maintained by 3 m*M* GSH (Fig. 3A). The results of Fig. 3 represent a stable steady state of enzyme reaction rate obtained by a 15-min preincubation of enzyme under the indicated GSH:GSSG ratios prior to initiation of assay by substrate addition. In separate experiments, a stable inhibitory effect was attained within 1–2 min when GSSG was added to cathepsin B previously activated by 2–5 m*M* GSH. In cathepsin B reactions activated over the range of 2–5 m*M* GSH, the addition of equimolar 2–5 m*M* GSSG concentrations (*i.e.,* GSH:GSSG = 1:1) caused ~90% inhibition of the reaction rates. Stability of all GSH:GSSG redox buffering

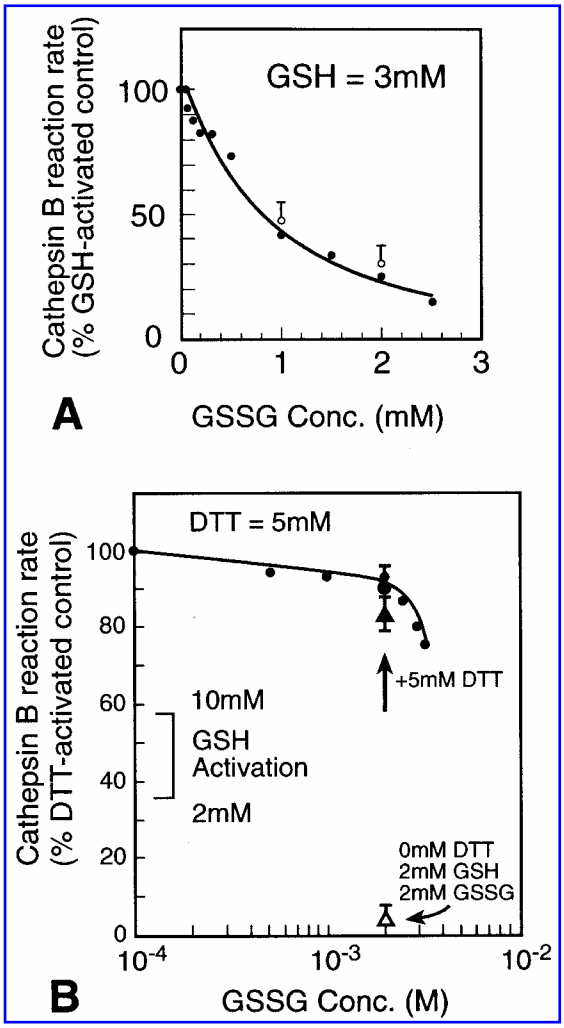

FIG. 3. (A) Effect of GSSG concentration on cathepsin B reaction rate activated by 3 m*M* GSH. Cathepsin B was preliminarily incubated for 15 min in buffer at the indicated GSH/GSSG concentrations prior to initiation of the reaction by addition of substrate. The 100% control reaction contained 3 m*M* GSH without GSSG. **(B)** Effect of GSSG concentration on cathepsin B reaction rate activated by 5 m*M* DDT. GSSG was combined with enzyme in buffer 20 min prior to addition of 5 m*M* DTT followed in 2 min by substrate. Constant reaction rates were then measured over 15 min following initiation. The 100% control value represents the DTT-activated reaction with no GSSG, which was indistinguishable from that with 10^{-4} *M* GSSG. Comparative activation by the 2–10 m*M* range of GSH concentration versus DTT is indicated as described in Fig. 2. At concentrations below 2 m*M*, GSSG did not appreciably inhibit the protease activated with 5 m*M* DTT. GSSG (2 m*M*) almost completely inhibited the reaction activated by 2 m*M* GSH (open triangle ± SD). Addition of 5 m*M* DTT to identical GSSG (2 m*M*)-inhibited reactions reactivated the enzyme within 4 min almost to the rate observed without GSSG (filled triangle ± SD). DTT (5 m*M*) caused little reactivation of cathepsin B previously inactivated by 10 m*M* GSSG when assayed after a 0.5 h preincubation period.

was verified by preincubation of enzyme with redox buffers for 1 h, followed by observation of stable reaction rates for a subsequent 1-h period.

Endogenous glutathione concentration maintains a submaximal cysteine protease reaction rate, which can be further activated four- to fivefold by DHLA

The steady-state submaximal cathepsin B reaction maintained by 2 m*M* GSH was arbitrarily selected as the 100% reference reaction rate for comparisons with redox activation and inhibition with DHLA and lipoamide disulfide, respectively. The separate individual actions of GSH and DHLA (Fig. 2) were approximately additive up to the maximal possible activation of cathepsin B (Figs. 4 and 5A). DHLA exhibited ~1,000-fold greater activation potency than GSH (Fig. 2) and four- to fivefold greater maximal activation than the reference reaction rate maintained by 2 m*M* GSH (Fig. 4).

GSSG reverses activation of cathepsin B caused by GSH, but not activation caused by excess DTT or DHLA

Although GSSG opposed the activation of cathepsin B caused by GSH (Fig. 3A), similar GSSG concentrations did not inhibit cathepsin B when activated by excess amounts of the stronger reductant DTT (Fig. 3B). AGSSG concentration range of 0.1–2 m*M* caused only several percent inhibition of the cathepsin B reaction rate previously activated by excess 5 m*M* DTT. Increasing GSSG concentration did not appreciably reverse the activation of cathepsin B by 5 m*M* DTT until stoichiometric equivalence was approached near 5 m*M* GSSG. Excess DHLA (2 m*M*) also overcame the inhibitory effect of GSSG (1 m*M*) on GSH (2 m*M*)-activated cathepsin B activity (data not shown). Because excess GSSG oxidizes DTT and DHLA (21, 22), it was predicted and confirmed that a 1-h preincubation of cathepsin B under 10–100 μ*M* DHLA or 0.5 m*M* DTT added to prior excess 2 m*M* GSSG resulted in completely inactive protease (data not shown).

These interactions with disulfhydryl agents imply that the inhibitory action of GSSG is attributable to a redox phenomenon, and not a competition of GSSG peptide for cathepsin B substrate binding sites. When excess 5 m*M* DTT was added to submaximal cathepsin B reactions ongoing at a GSH:GSSG ratio of 2 m*M*:2 m*M*, the reactions were activated within 1–2 min to rates near those observed under DTT alone (Fig. 3B). Excess DTT can eliminate GSSG by reduction to GSH over ~30 min; however, the time course of GSSG reduction is much longer than the immediate reversal of GSSG protease inhibition (18, 19). Thus, the immediate onset of protease reactivation by disulfhydryl agents is much faster than the reductive elimination of GSSG by DTT. Indeed, GSSG (1 m*M*) caused no transient inhibition when added to ongoing cathepsin B reactions previously activated maximally by 5 m*M* DTT (data not shown). Importantly, GSSG is known also to inhibit redox-responsive enzymes other than proteases where peptide–peptide substrate competition does not occur (11). Finally, the protease inhibitory action of oxidized lipoic acid is not associated with a peptide structure, as described below.

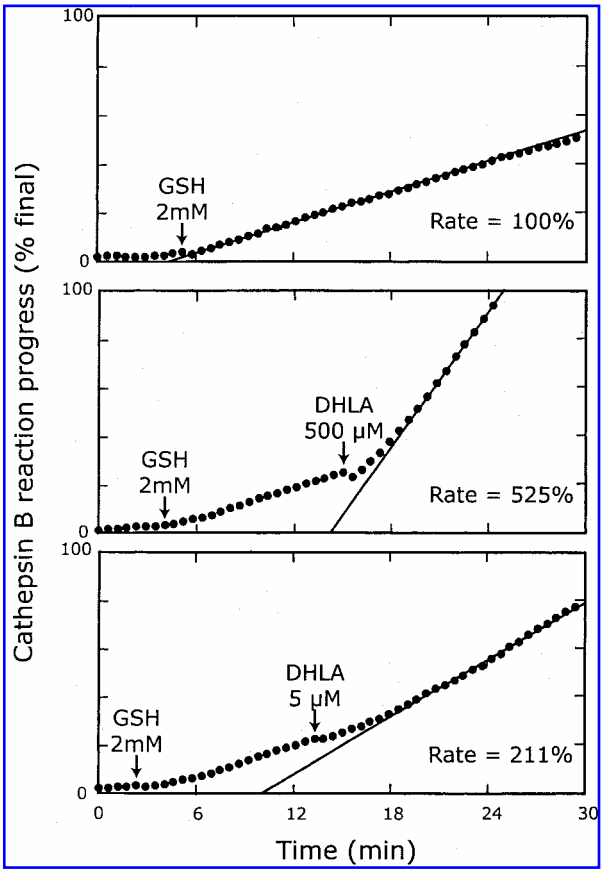

FIG. 4. Activation of cathepsin B with GSH and combined GSH with DHLA. Cathepsin B was previously inactivated as in Fig. 1. The identical amount of inactivated cathepsin B was simultaneously added to buffer and substrate in three reaction wells prior to the time shown. At the times indicated by the arrows, GSH (2 m*M*) was added to each well resulting in maintenance of a steady-state submaximal activation. DHLA (5 or 500 μ*M* here; or 50 μ*M* in Fig. 5A) was subsequently added at the times indicated. The rates of reactions are described by the slopes of reaction progress. Percent changes in reaction rates were calculated using the fitted measurements of individual reaction slopes before and after DHLA addition. Similar results were confirmed using substrate concentrations of 10 and 100 μ*M* in place of 25 μ*M*. The submaximal actions of GSH and DHLA were essentially additive and did not exceed the maximal action of DHLA alone. The DHLA activations added to GSH controls are 246 ± 31% and 482 ± 64% for 5 and 500 μ*M*, respectively, and 435 ± 60% for 50 μ*M* (Fig. 5A), (means ± SD, $p < 0.01$).

Lipoic acid disulfide [LA(S-S)] opposes GSH activation of cathepsin B with greater potency than GSSG

Experiments using aged preparations of lipoic acid unexpectedly indicated that the oxidized disulfide form was inhibitory to reductive activation of protease by lower amounts of DHLA at some unknown redox ratio. After 24–48 h of storage in DMSO, largely oxidized preparations of lipoic acid

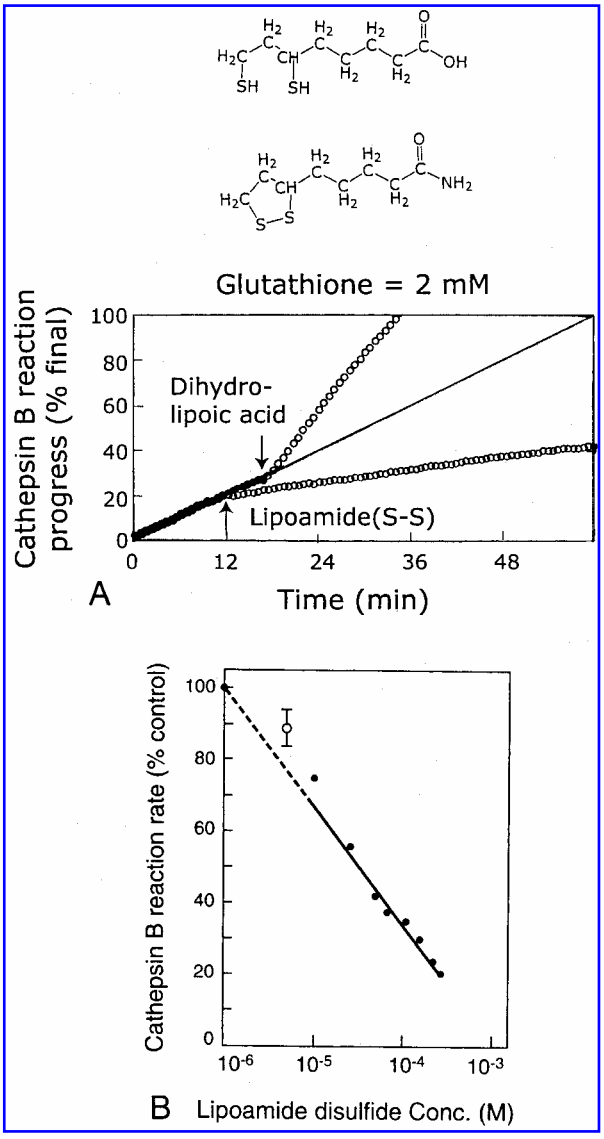

FIG. 5. **(A)** Steady-state activation of cathepsin B reaction by endogenous GSH concentration, further activation by DHLA, and inhibition by lipoamide disulfide. Cathepsin B was preliminarily activated to constant reaction rate by exposure to GSH (2 m*M*) as in Fig. 4. The progress of two separate reactions was statistically fitted (•) and is represented by the diagonal extrapolation. At the indicated times, 50 μ*M* of DHLA (top structure) or lipoamide disulfide (bottom structure) was added to the ongoing reactions. The difference between inhibited and activated reaction slopes represents nearly a 10-fold kinetic span above and below the basal rate maintained by 2 m*M* GSH. **(B)** Effect of lipoamide disulfide concentration on cathepsin B reaction rate previously activated by 2 m*M* GSH. The 100% value represents the steady-state reaction rate under 2 m*M* GSH alone as shown by the slope in A. The indicated concentrations of lipoamide disulfide were added to ongoing steady-state reactions activated by GSH 15 min previously. Values shown are constant steady-state inhibitions. At lipoamide disulfide concentrations below 10 μ*M* (extrapolated dashed trace), the inhibition was delayed by ~10–15 min as illustrated at 5 μ*M* from separate experiments (see text) (open circle ± SD).

yielded a biphasic protease activation profile having much less peak activation with decreased optimum near 100–200 μ*M*, and little activity near either 10 μ*M* or 1 m*M* (data not shown). Accordingly, when fresh DHLA preparations are used, the submaximal cathepsin B activation observed above the peak at 1 m*M* (Fig. 1) might be partly attributable to unavoidable initial contamination with oxidized LA(S-S).

The oxidized dithiolane ring of LA(S-S) is less soluble than the disulfhydryls of DHLA; therefore, the amide derivative of LA(S-S) is generally used for aqueous experimentation (Fig. 5). In experiments similar to Fig. 1, inclusion of equal lipoamide disulfide concentration with fresh DHLA in a range near 5 μ*M* did not alter the activating action of DHLA alone (data not shown). However, differing solubilities of disulfhydryls and the oxidized dithiolane ring complicate direct comparison of concentrations.

Lipoamide disulfide (20–250 μ*M*) caused a prompt inhibition of cathepsin B previously activated by 2 m*M* GSH (Fig. 5). However, determination of an exact threshold of lipoamide disulfide action was complicated by a 10–15-min time lag for the onset of minimal protease inhibition below 10 μ*M* (Fig. 5A). When lipoamide disulfide concentrations of 20 μ*M* or greater were added to GSH-activated cathepsin B reactions, a decline in rate began promptly and approached a decreased steady state in ~2 min (Fig. 5A). The actual experimental threshold of this inhibitory lipoamide disulfide action was ~5 μ*M*, which caused ~5–10% delayed inhibition of GSH-activated cathepsin B activity over 10-15min (data superimposed in Fig. 5B at 5 μ*M*). This delay of threshold lipoamide disulfide inhibitory action is presumably due to diffusion limitation of an insoluble agent. Despite all experimental uncertainties, reduced DHLA and oxidized lipoamide disulfide are far more potent as protease activator and inhibitor than GSH and GSSG, respectively.

Basal intracellular protein degradation functions at a rate intermediate between pro-oxidative decreases and pro-reductive increases

The averaged basal rate of bulk protein degradation in various *in vivo* tissues or cultured cell types is ~2–3%/h corresponding to turnover half-times of 24–35 h (25). Due to incomplete labeling of slow turnover proteins, the present rate of release of [^{3}H]leucine from macromolecular form is ~3.5%/h observed from 3–5 h postlabeling (25).

From 0–3 hr postlabeling, [^{3}H]leucine is released largely from the well-known ER-Golgi subcomponent. Rapid turnover proteolysis comprises 5% of total cell protein content, which is degraded with a half-time of ~45 min and largely eliminated by 3 h. The pro-oxidant, diamide, inhibits all of the rapid turnover degradative subcomponent (23, 24). However, elevations in rapid turnover proteolysis are technically difficult to measure and interpret accurately, and were not characterized in the present studies.

From 3–5 h postlabeling, total remaining [^{3}H]leucine release can be experimentally partitioned into two redox-responsive and one redox-unresponsive subcomponents, *i.e.,* three remaining experimental subcomponents (Fig. 6A). In Fig. 6, the 100% control represents the normalized rate of

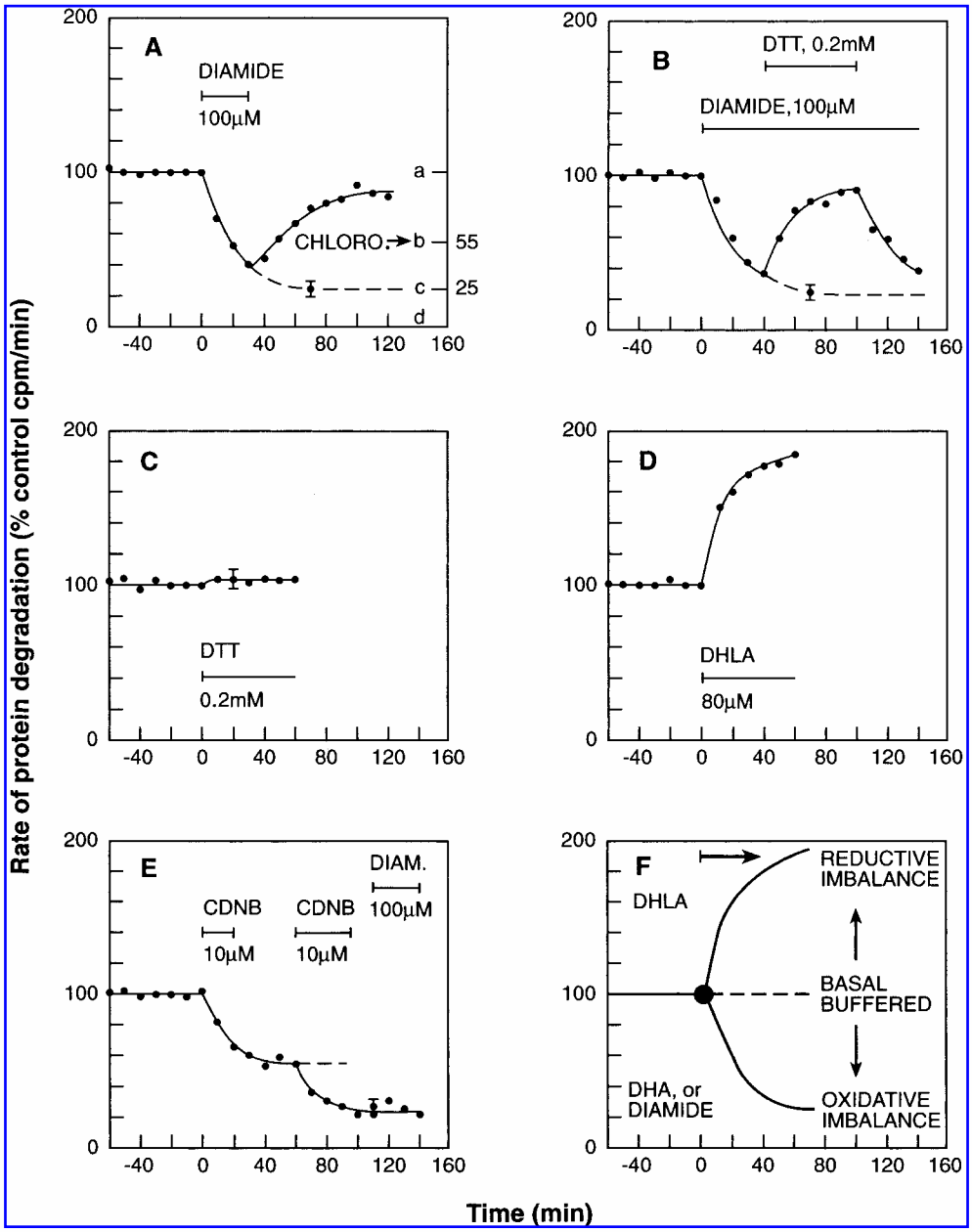

FIG. 6. Redox responsiveness of basal protein degradation in the contractile perfused rat heart. Experimental design, characteristics of the preparation, and statistical fitting of the indicated traces have been previously described (23, 24). [^{3}H]Leucine release from biosynthetically labeled proteins was measured at 2-min intervals in continuously collected nonrecirculating perfusate. The means of five measurements are shown at 10 min intervals. In all panels, a 3-h preliminary degradation period preceded zero time (see Experimental Procedures). Experimental agents were infused from 100–1,000-fold concentrated solutions into nonrecirculating perfusate over the time periods indicated. Measurements shown (± SD) are significantly different from extrapolated 100% control baselines normalized to SD = 0 ($p \ll 0.01$, $n = 3$ or 4 separate hearts). All results are representative of at least three similar experiments. **(A)** Spontaneously reversible inhibition of basal protein degradation with diamide (100 μ*M*). At 3 h postlabeling, three remaining subcomponents can be distinguished as described previously (24, 25; see text). One hundred percent of basal protein degradation includes 75% that is inhibitable by diamide (redox-responsive) (a–c) and 25% that is diamide-uninhibitable (redox-unresponsive)(c–d). Chloroquine inhibits the lysosomal pathway consisting of 45% (a–b). The 75% inhibition caused by diamide includes the 45% chloroquine-sensitive lysosomal pathway. The difference between diamide action and chloroquine action defines an extravesicular subcomponent of unknown cellular distribution: 75% − 45% = 30%, (b–c). The b–c subcomponent is referred to as extralysosomal, redox-responsive. **(B)** Reversal of diamide inhibition of proteolysis by simultaneous infusion of excess DTT. **(C)** Absence of effect of 0.2 m*M* DTT on basal proteolysis. **(D)** Elevation of basal protein degradation caused by DHLA (80 μ*M*). **(E)** Effect of interrupted CDNB infusion on protein degradation. Nonadditive inhibition caused by combined diamide and CDNB is shown ± SD ($p > 0.1$). **(F)** Summary of the kinetics of redox-responsive [^{3}H]leucine release from myocardial proteins.

[^{3}H]leucine release following elimination of rapidly degraded proteins during a 3-h preliminary degradation period (see Experimental Procedures). At any time 3–5 h postlabeling, chloroquine maximally inhibits 45% of total proteolysis consisting of the lysosomal subcomponent (Fig. 6A, a-b). From 3–5 h postlabeling, diamide reversibly inhibits 75% of proteolysis, including the lysosomal subcomponent and an extravesicular redox-responsive subcomponent (Fig. 6A, a-c). Approximately 25% of total proteolysis is not inhibited by combined chloroquine and diamide, and this is defined as the nonlysosomal redox-unresponsive subcomponent (Fig. 6A, c-d). From 3–5 h postlabeling, an extralysosomal redox-responsive subcomponent of 30% is experimentally defined by subtraction of the 45% inhibition caused by chloroquine from the 75% inhibition caused by diamide (Fig. 6A, b-c; see also 23–25). ER-Golgi and lysosomal proteolysis represent spatially defined vesicular compartments; however, the extravesicular subcomponents are presumably distributed throughout the cell. The experimental division of extravesicular proteolysis into distinct redox-dependent and redox-independent subcomponents presumably represents maximal experimental responsiveness of diverse processes not normally inhibited completely *in vivo*.

Simultaneous infusion of diamide and DTT caused antagonistic opposing actions with the predominant effect mediated by the agent present in stoichiometric excess. The proteolytic inhibitory action of diamide (100 μM) can be reversed by stoichiometric excess of DTT (200 μM) under simultaneous infusion of both agents (Fig. 6B). DTT is known to rapidly reduce both diamide and protein-glutathione disulfides (25).

Results indicate that basal cell protein degradation proceeds at an intermediate rate that can be further activated by reductive agents or inhibited by oxidative agents (Fig. 6). DTT caused little elevation in basal cell protein degradation at infused 0.2mM concentrations, which did cause partial activation of cathepsin B assay shown below (Figs. 2 and 6C). It cannot be determined whether most of 0.2 mM infused DTT enters the cell without changing proteolysis or is rather oxidized extracellularly. A hypothetical surviving permeant DTT concentration below 50 μM would correspond to the steep portion of the concentration-response curve of Fig. 2. The action of endogenous reductive activators might overlap the action of low amounts of DTT in unknown cell compartments. The basal rate of cell protein degradation could be increased by sufficient higher amounts of DTT; however, the maximal intracellular DTT action could not be determined due to DTT toxicity at submaximally effective concentrations (data not shown). DTT concentrations of 5 mM are typically required to maximally activate most sulfhydryl-dependent enzymes; and 15–20 mM DTT is typically used to reduce intramolecular protein disulfides. Infusion of 2.0 mM DTT caused prompt decrement in contractile function, severely weakened dysrhythmia, and death. Much of DTT injury is attributable to its weak acidity (pK_a = 3.3). Weak acids at 1–2 mM concentrations partially defeat Krebs bicarbonate buffer and cause acidosis. This preparation is injured at extracellular pH 7.0 and dies rapidly at pH 6.8.

DHLA elevated myocardial proteolysis with far greater potency than DTT at DHLA concentrations that did not defeat the Krebs bicarbonate buffer. DHLA at 80 μM caused an 80–90% elevation of protein degradation beginning promptly (Fig. 6D). Most of DHLA action could be prevented or reversed by excess of diamide, although not rigorously characterized. Elevation of intracellular protein degradation under constant extracellular DHLA infusion was not merely concentration-dependent, but also proportional to the time of exposure (data not shown). Under constant extracellular exposure, concentration–time-dependent actions are typical of drugs that either bind irreversibly to their targets or accumulate at targets or lipid depots due to hydrophobicity. A sustained 200 μM DHLA exposure under present vascular perfusion caused eventual myocardial contractile decrement increasing after 1 h (7).

Unanticipated results with DTT and DHLA suggest that substrate protein reduction and unfolding cannot account for most of the elevation in proteolysis under disulfhydryl exposure (see also 15). In injured preparations, basal proteolysis can decline prior to death, although without correlation with measured ATP levels (24, 25). The elevation of basal protein degradation exhibited a biphasic DTT concentration dependence, in association with cell injury near 2 mM DTT. The maximal possible elevation in bulk proteolysis under infused DTT was limited to ~30–40% near 2 mM, and progressively declined by half as the preparation was injured under continuous DTT exposure. A similar biphasic proteolytic elevation was noted under injurious DHLA concentration higher than that causing sustained elevations (Fig. 6). If the disulfhydryl-induced proteolytic elevation (Fig. 6) were caused exclusively by reductive substrate unfolding, then the elevation could not decline under higher injurious disulfhydryl exposure (see Discussion). The effect of physiologically relevant lipoamide disulfide infusion on intracellular proteolysis was not attempted in view of metabolic reduction, a likely biphasic concentration dependence, and the uncertainty of accumulation kinetics as described above (1, 6, 14). Identification of pathways involved in DHLA-activated proteolysis was not attempted due to interpretive limitations inherent in the use of inhibitors for present purposes.

Intracellular protein degradation is irreversibly inhibited by a substrate for glutathione transferase known to deplete GSH by covalent conjugation

1-Chloro-2,4-dinitrobenzene (CDNB) is widely used to deplete mammalian tissue GSH because it is rapidly conjugated by enzymatic action of glutathione transferases (16, 17). CDNB noninjuriously depletes most GSH in the perfused rat heart in less than 1 h (17).

CDNB inhibited the same proteolytic subcomponents as did diamide; however, the kinetics of their actions differed markedly (Fig. 6E). As predicted by its known mechanism, CDNB antiproteolytic action was not concentration-dependent, but rather concentration–time-dependent. Interruption of CDNB infusion (10 μM) caused interruption of the progress of proteolytic inhibition without reversal. Upon continuation of interrupted CDNB infusion, protein degradation proceeded to maximal inhibition of 75%. Exposure to higher 100 μM CDNB caused the maximal 75% proteolytic inhibition in ~10 min (data not shown). Simultaneous infusion of diamide (100 μM) with CDNB did not cause additive prote-

olytic inhibition greater than the maximal 75% inhibition caused by either agent alone (Fig. 6E). Under exposure to low CDNB concentrations, this tissue did not exhibit a sustained threshold against proteolytic inhibition, but rather a delay of onset followed by slower progress to the maximal final action. When only 1 μM CDNB was infused, the progress of inhibition began after a lag of 5–10 min, then proceeded slowly to the maximal 75% inhibition over ~2 h (data not shown). Proteolysis was inhibited by CDNB without simultaneous contractile dysfunction, although later toxicity is likely.

Diamide promotes reversible formation of disulfides, but does not form irreversible covalent adducts (22). In contrast to CDNB, the reversible action of diamide exhibited a true threshold of several micromolar, which this tissue could sustain over 1–2 h or indefinitely without inhibition of proteolysis (data not shown). As previously illustrated, prolonged 10 μM diamide exposure caused a slight proteolytic inhibition that proceeded very gradually to 15% over 1–2 h (see 24). When diamide was infused at 1 mM concentration, 10-fold above maximal 100 μM concentration, the 75% inhibition of proteolysis was irreversible after a 40-min exposure, perhaps in association with export of oxidized glutathione (17) and/or oxidative injury.

DISCUSSION

Both cysteine protease reaction rates *ex vivo* and much of intracellular proteolysis appear to be graded in response to concentrations, redox ratios, and individual reductive potentials of multiple interactive redox mediators. Basal cell protein degradation is poised at a submaximal rate intermediate between prooxidative inhibition and pro-reductive activation. The kinetic limits of nonpathogenic changes in steady-state protein replacement are unknown, but presumably less than the ±80% shown in Fig. 6F. The relative contributions of various cysteine proteases to the present results remain to be determined; however, multiple activities are presumably recruited at various DHLA concentrations. Present cathepsin B was preliminarily processed from the pro-enzyme to fully mature enzyme prior to all assays. Reversible redox modulation of protease reaction rate or intracellular proteolysis cannot be attributable to control by cleavage of pro-regions. Nonetheless, some amount of DHLA-activated intracellular protein degradation might be increased secondary to cleavage of unidentified pro-proteases to mature proteases (31, 36).

DHLA action on cell protein degradation might include some unknown combination of reductive substrate unfolding and activation of multiple cysteine proteases directly or via thioredoxin (15).

Previously described redox reactions activating cysteine proteases

Three well known protein reductive reactions have been previously distinguished in defined cysteine protease assays (2, 9, 11, 20, 25, 27, 36, 37, 38)

(A) $$PrS_{ox} + 2\ GSH \leftrightarrow PrS_{red} + GSSG$$

(B) $$PrS_{ox} + LA(SH)_2 \leftrightarrow PrS_{red} + LA(S\text{-}S)$$

or

DTT

or

thioredoxin $(SH)_2$

(C) $$PrS\text{-}SR + GSH \leftrightarrow PrSH + GS\text{-}SR$$

PrS_{ox} represents many reversible protein sulfur oxidation products including oxygenations, nitrosations, metal ion binding, covalent interactions with many pro-oxidative metabolites, disulfide formation, and others. Reaction A can provide graded responsiveness of cathepsin B reaction in relation to GSH concentration. Under low GSSG concentration, there is little back reaction, and the driving potential for reduction of trace amounts of protease by coupling to 2 GSH oxidation is dependent primarily on the concentration of GSH. Glutaredoxins can catalyze nonspecific reductions coupled to the 2GSH oxidation with unknown relationship to the present results (2, 27). Reaction B involves coupling of protease reductions with the intramolecular oxidation of DTT, DLHA [indicated here as LA(SH)2)], or thioredoxin disulfhydryls to their respective intramolecular disulfides (19, 38). Due to the large change in free energy upon intramolecular oxidation of vicinal disulfhydryls, the back reactions are small after reduction of electron acceptors such as GSSG (18). Accordingly, when DHLA is mixed in equimolar amounts with GSSG *ex vivo*, the intramolecular oxidation to LA(S-S) drives reduction of GSSG to 2 GSH nearly to completion with little back reaction (18). Reaction C is an exchange of redox status between a protein heterodisulfide and a surrounding monosulfhydryl. GSH exchange activation has been demonstrated with the artificial papain heterodisulfide formed from methylmethane thiosulfonate: papain-S-SCH_3 (37).

GSH:GSSG redox buffering of protein functions in defined assay

According to the simplified model, a protein can exist as a redox couple in equilibrium with the surrounding GSH:GSSG redox status via reversible formation of a mixed protein–SG sulfide linkage (forward and back reactions in C above). The equilibrium fraction of inactive enzyme increases in proportion to the fraction of protein heterodisulfide adduct (PrSH)/(PrSSG), which is proportional to the surrounding GSH:GSSG redox ratio (for reviews, see 9, 11).

$$\frac{[PrSH]}{[PrSSG]} = K\,\frac{[GSH]}{[GSSG]}$$

The redox responsiveness of individual enzyme sulfhydryls can differ markedly in association with accessibility, surrounding interactive structures, pK_a, metal interactions, etc. (11). Separate studies indicate that some recombinant L-cathepsins are uninhibited by GSSG concentrations that markedly inhibit cathepsin B under identical conditions, *e.g.,* cruzain or falcipain-2. Experimentation with redox buffers

does not clearly distinguish covalent protein heterodisulfide formation from an enzyme inhibitory effect of the stable independent disulfide bond. Second, a protein sulfhydryl might form multisulfide bonds with more than one nonprotein sulfhydryl as reviewed previously (11). Third, multiple protein redox sites on the same protein are also conceivable, *e.g.,* reactive site amino acids, internal disulfides, surface monosulfhydryls, bound metal ions.

In microbes, the GSH:GSSG redox ratio can indeed vary markedly under metabolic shifts. However, mammalian cells normally maintain major redox couples 99% reduced. Although the mammalian intracellular GSH:GSSG redox ratio is defended, the total reduced GSH concentration can vary markedly as part of normal cell function, *e.g.,* diurnal fluctuations (9). Present results indicate that the reduced GSH concentration might be a more important protease controlling factor than the GSH:GSSG redox ratio, except under gross prooxidative metabolic shifts. Present results do not demonstrate that the dithiolane ring of LA(S-S) is a significant cathepsin B inhibitor at likely endogenous levels (Fig. 5). Nonetheless, *in vivo* and *ex vivo* correspondences are unknown. Interestingly, some oxidized thioredoxins are suggested to serve as protein oxidants under some circumstances (8).

Lipoic acid as a pharmacologic or endogenous modulator of cysteine proteases

Cells can export and import pharmacologically administered lipoic acid. Oxidized lipoamide disulfide taken up by cells can be readily reduced by either thioredoxin reductase or glutathione reductase (1, 6, 14). Speculatively, either protein-ligated or nonbound endogenous DHLA could serve in the activation of proteolysis (15, 23, 40). A proteolytic controlling role of DHLA could be associated with direct DHLA reduction of protease active sites and/or indirect reduction of intermediaries that might influence protease active sites.The exact endogenous DHLA concentration is uncertain. However, mitochondria normally undergo extensive fusions with the lysosomal vacuolar system, thereby creating a partitioned proteolytic compartment with concentrated DHLA (9, 13, 39).

Redox-responsive protein degradation could conceivably enhance or limit the many functions served by cysteine proteases in diverse cell types. For example, recent studies suggest that supraphysiologic reductive stress might be among pro-apoptotic factors (28, 36), and that cathepsin and calpain-mediated proteolysis might contribute to apoptogenesis (4, 10, 28). Many targets might permit development of pharmacologic intervention against excess or insufficiency of redox-responsive proteolysis in various applications (34).

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ABBREVIATIONS

AMC, 7-amino-4-methylcoumarin; CDNB, 1-chloro-2,4-dinitrobenzene; DHLA, dihydrolipoic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GSH, glutathione; GSSG, glutathione homodisulfide; LA(S-S), lipoic acid disulfide.

REFERENCES

1. Arner ES, Nordberg J, and Holmgren A. Efficient reduction of lipoamide and lipoic acid by mammalian thioredoxin reductase. *Biochem Biophys Res Commun* 225: 268–274, 1996.
2. Biaglow JE, Donahue J, Tuttle S, Held K, Chrestensen C, and Mieyal J. A method for measuring disulfide reduction by cultured mammalian cells: relative contributions of glutathione dependent and glutathione independent mechanisms. *Anal Biochem* 281: 77–86, 2000.
3. Biggs JR, Yang J, Gullberg U, Muchardt C, Yaniv M, and Kraft DS. The human brm protein is cleaved during apoptosis: the role of cathepsin G. *Proc Natl Acad Sci U S A* 98: 3814–3819, 2001.
4. Blomgren K, Zhu C, Wang X, Karlsson JO, Leverin AL, Bahr BA, Mallard C, and Hagberg H. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia–ischemia: a mechanism of "pathological apoptosis"? *J Biol Chem* 276: 10191–10198, 2001.
5. Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ 3rd Charrier V, and Parsonage D. Protein sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38: 15407–15416, 1999.
6. Constantinescu A, Pick U, Handelman GJ, Haramaki N, Han D, Podda M, Tritschler HJ, and Packer L. Reduction and transport of lipoic acid by human erythrocytes. *Biochem Pharmacol* 50: 253–261, 1995.
7. Coombs JS, Powers SK, Rowell B, Hamilton KL, Dodd SL, Shanely RA, Sen CK, and Packer L. Effects of vitamin E and alpha-lipoic acid on skeletal muscle contractile properties. *J Appl Physiol* 90: 1424–1430, 2001.
8. Debarbieux L and Beckwith J. On the functional interchangeability, oxidant vs. reductant, of members of the thioredoxin superfamily. *J Bacteriol* 182: 723–727, 2000.
9. Deneke SM. Thiol-based antioxidants. *Curr Top Cell Regul* 36: 151–180, 2000.
10. Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M, and Jaattela M. Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. *J Cell Biol* 153: 999–1010, 2001.
11. Gilbert HF. Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol* 251: 8–28, 1995.
12. Gueguen V, Macherel D, Jaquinod M, Douce R, and Bourguignon J. Fatty acid and lipoic acid biosynthesis in higher plant mitochondria. *J Biol Chem* 275: 5016–5025, 2000.
13. Haj-Yehia AT, Assaf P, Nassar T, and Katzhendler J. Determination of lipoic acid and dihydrolipoic acid in human plasma and urine by high performance liquid chromatography with fluorimetric detection. *J Chromatogr* 18: 381–388, 2000.
14. Handelman GJ, Han D, Tritschler H, and Packer L. Alpha-lipoic acid reduction by mammalian cells to the dithiol form and release into the growth medium. *Biochem Pharmacol* 47: 1725–1730, 1994.

15. Holmgren A. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem* 254: 9627–9632, 1979.
16. Huang D, Xiao H, Samii JM, Vila JA, and Keaney JF Jr. Contrasting effects of thiol modulating agents on endothelial NO bioactivity. *Am J Physiol* 281: C719–C725, 2001.
17. Ishikawa T and Sies H. Cardiac transport of glutathione disulfide and S-conjugate: studies with isolated perfused rat heart during hydroperoxide metabolism. *J Biol Chem* 259: 2838–2843, 1984.
18. Jocelyn PC. The standard redox potential of cysteine-cystine from the thiol-disulfide exchange reaction with glutathione and lipoic acid. *Eur J Biochem* 2: 327–331, 1967.
19. Jocelyn PC. Chemical reduction of disulfides. *Methods Enzymol* 143: 246–256, 1987.
20. Kerblat I. Importance of thioredoxin in the proteolysis of an immunoglobulin G as antigen by lysosomal cysteine proteases. *Immunology* 97: 62–68, 1999.
21. Klatt P and Lamar S. Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944, 2000.
22. Kosower NS and Kosower EM. Diamide: an oxidant probe for thiols. *Methods Enzymol* 25: 123–133, 1995.
23. Lockwood TD. Inactivation of intracellular proteolysis and cathepsin B enzyme activity by dehydroascorbic acid and reactivation by dithiothreitol in perfused rat heart. *Biochem Pharmacol* 54: 669–675, 1997.
24. Lockwood TD. Redox dependent and redox independent subcomponents of protein degradation in perfused myocardium. *Am J Physiol* 276: 945–954, 1999.
25. Lockwood TD. Redox control of protein degradation. *Antioxid Redox Signal* 2: 851–878, 2000.
26. Morris TW, Reed KE, and Cronan JE Jr. Lipoic acid metabolism in *E. coli:* the lip A and lip B genes define redundant pathways for ligation of lipoyl groups to apoproteins. *J Bacteriol* 177: 1–10, 1999.
27. Packer L. (Ed.) Biothiols Part A: monothiols and dithiols, protein thiols and thiyl radicals. *Methods Enzymol* 251, 1995.
28. Park HS, Huh SH, Kiam Y, Shim J, Lee SH, Park IS, Jung YK, Kim IY, and Choi EJ. Selenite negatively regulates caspase-3 through a redox mechanism. *J Biol Chem* 275: 8487–8491, 2000.
29. Perham RN. Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. *Annu Rev Biochem* 69: 961–1004, 2000.
30. Phan UT, Arunachalam B, and Cresswell P. Gamma-interferon-inducible lysosomal thiol reductase (GILT): maturation, activity and mechanism of action. *J Biol Chem* 275: 25907–25914, 2000.
31. Quraishi O and Storer AC. Identification of internal autoproteolytic cleavage sites within the prosegments of recombinant procathepsin B and procathepsin S: Contribution of a plausible unimolecular autoproteolytic event for the processing of zymogens belonging to the papain family. *J Biol Chem* 276: 8118–8124, 2001.
32. Reed LJ. A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. *J Biol Chem* 276: 38329–38336, 2001.
33. Salvador N, Aguado C, Horst M, and Knecht E. Import of a cytosolic protein into lysosomes by chaperone-mediated autophagy depends on its folding state. *J Biol Chem* 275: 27445–27456, 2000.
34. Self WT, Tsai L, and Stadtman TC. Synthesis and characterization of selenotrisulfide-derivatives of lipoic acid and lipoamide. *Proc Natl Acad Sci U S A* 97: 12481–12486, 2000.
35. Sen CK. Cellular thiols and redox regulated signal transduction. *Curr Top Cell Regul* 36: 1–30, 2000.
36. Sen CK, Roy S, and Packer L. Fas-mediated apoptosis of human Jurkat T-cells: intracellular events and potentiation by redox-active α-lipoic acid. *Cell Death Differ* 6: 481–491, 1999.
37. Singh R, Blater WA, and Collison AR. Thiol reactivation assay based upon reactivation of papain. *Methods Enzymol* 251: 229–237, 1995.
38. Stephen AG, Powls R, and Beynon RJ. Activation of oxidized cysteine proteinases by thioredoxin-mediated reduction in vitro. *Biochem J* 291: 345–347, 1993.
39. Wada H, Shintani D, and Ohlrogge J. Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proc Natl Acad Sci U S A* 94: 1591–1596, 1997.
40. Xu DP and Wells WW. Alpha-lipoic acid dependent regeneration of ascorbic acid from dehydroascorbic acid in rat liver mitochondria. *J Bioenerg Biomembr* 28: 77–85, 1996.

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2. Rintaro YAMANISHI. 2009. *KAGAKU TO SEIBUTSU* **47**:11, 764-771. [CrossRef]
3. Emmanuel Godat, Virginie Hervé-Grépinet, Florian Veillard, Fabien Lecaille, Maya Belghazi, Dieter Brömme, Gilles Lalmanach. 2008. Regulation of cathepsin K activity by hydrogen peroxide. *Biological Chemistry* **0**:ja, 080808064244425-15. [CrossRef]
4. Emmanuel Godat, Virginie Hervé-Grépinet, Florian Veillard, Fabien Lecaille, Maya Belghazi, Dieter Brömme, Gilles Lalmanach. 2008. Regulation of cathepsin K activity by hydrogen peroxide. *Biological Chemistry* **389**:8, 1123-1126. [CrossRef]
5. Saki TAKEDA, Noriko BANDO, Rintaro YAMANISHI. 2008. Ingested #-Carotene Enhances Glutathione Level and up-Regulates the Activity of Cysteine Cathepsin in Murine Splenocytes. *Bioscience, Biotechnology, and Biochemistry* **72**:6, 1595-1600. [CrossRef]
6. T LOCKWOOD. 2007. Is dihydrolipoic acid among the reductive activators of parasite CysHis proteases?. *Experimental Parasitology* . [CrossRef]
7. Thomas D. Lockwood . 2005. The Transfer of Reductive Energy and Pace of Proteome Turnover: A Theory of Integrated Catabolic Control. *Antioxidants & Redox Signaling* **7**:7-8, 982-998. [Abstract] [Full Text PDF] [Full Text PDF with Links]
8. Thomas D. Lockwood. 2004. Cys-His proteases are among the wired proteins of the cell. *Archives of Biochemistry and Biophysics* **432**:1, 12-24. [CrossRef]
9. Adrian T. McCollum, Faegheh Jafarifar, Roy Chan, Rodney P. Guttmann. 2004. Oxidative stress inhibits ionomycin-mediated cell death in cortical neurons. *Journal of Neuroscience Research* **76**:1, 104-109. [CrossRef]
10. Thomas D Lockwood. 2003. Redox pacing of proteome turnover: influences of glutathione and ketonemia. *Archives of Biochemistry and Biophysics* **417**:2, 183-193. [CrossRef]