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# Redox pacing of proteome turnover: influences of glutathione and ketonemia

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**Abstract**

In early starvation tissue protein degradation increases, however in later starvation proteolysis declines so as to pace gradual atrophy during synthetic failure. Secondary decline of proteolytic pathways under progressive nutritional desperation is unexplained. After several days of starvation tissue GSH is partly depleted and GSSG/GSH is increased, followed by onset of ketonemia from fat breakdown. Ketone bodies inexplicably delay net muscle protein loss. Recent studies identify a proteome subset of more than 200 proteins with reactive sulfhydryl sites as candidates for coordinate redox control of diverse cell functions. Ketones cause protein sulfhydryl oxidation and protein S-glutathionylation. Here, redox-responsive proteolytic pathways were bio-assayed by release of [$^3$H]leucine from rat myocardium under non-recirculating perfusion. More than 75% of myocardial protein degradation was inhibited and defined by infusion of diamide (100 μM) under constant physiologic concentrations of complete amino acids. Diamide-inhibitable proteolysis includes all lysosomal and some extra-lysosomal proteolysis. Following diamide washout, the reversal of proteolytic inhibitory action was greatly enhanced by artificial repletion of GSH by supra-physiologic extra-cellular GSH (1 mM) exposure. Therefore, GSH maintains much of constitutive protein degradation in a primary tissue bioassay. Physiologic acetoacetate infusion (5 mM) inhibited redox-responsive protein degradation. Uniformly [$^3$H]leucine labeled 3T3 cells exhibited similar redox-dependent and redox-independent subcomponents of protein degradation. Independent of ketones, steady state cathepsin B reaction rate ex vivo was graded in proportion to the GSH concentration without GSSG, and inversely proportional to the GSSG/GSH redox ratio with inhibitory threshold at 0.5% oxidized. Linkage of some cysteine protease reaction rates to the interplay between GSH-GSSG/GSH status and ketonemia is suggested among transcendent mechanisms coordinating and pacing proteome turnover under prolonged starvation. The possibility of pre-emptive, redox coordination of distinct proteolytic pathways is speculatively discussed.


*Keywords:* Cathepsin B; Glutathione; Acetoacetate; Proteolysis; Perfused heart; Amino acids

Survival under nutritional desperation was a major factor in mammalian evolution. Among first principles of metabolism is the conversion of proteome to energy during nutritional insufficiency. However, protein degradation is biphasic in early vs. late stages of starvation. Upon nutritional interruption, coordinate proteolysis initially increases in some tissues, so as to defend amino acid pools and protein synthesis over several days [1,2]. In many organisms and cell types, the advantages of protein turnover are secondarily compromised in order to postpone catastrophic protein loss under prolonged synthetic failure. Muscle protein degradation and synthesis both decline after several days of starvation, so as to pace the unbalanced turnover of gradual atrophy [2]. Studies of initial proteolytic activation mechanisms under early starvation have not addressed secondary inactivation mechanisms under later starvation. Explosive progress reveals the complexity of 500 proteases of the human genome and many individual proteolytic controls [3]. However, the integrative physiology of protein turnover must account for transcendent controls, which secondarily pre-empt other catabolic mechanisms during prolonged nutritional insufficiency.

After several days of starvation tissue GSH concentration decreases and GSSG/GSH ratio increases

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followed by onset of severe ketonemia (described below); however, the interplay between these distinct variables remains uncertain. Elevated fat breakdown provides preferential fuel from ketone bodies, thereby delaying amino acid catabolism and net protein loss in some tissues [4–7]. Under starvation or diabetes, ketone bodies can attain very high concentrations of 10–20 mM. Technical obstacles and uncontrolled covariables complicate in vivo measurements of circulating nutrients/metabolites, tissue redox status, and tissue proteolytic pathways as parameters of starvation time. Signals and pacing mechanisms linking biphasic proteolytic changes with the metabolism of starvation have not been studied in primary tissue under controlled exposure to nutrients/metabolites.

Hypotheses of biphasic proteolytic control during starvation cannot involve ATP depletion insofar as myocardial ATP is maintained in later stages. Most investigations of protein turnover during starvation have hypothesized a controlling role of amino acid pools that are somehow sensed by synthetic and degradative mechanisms. In yeast and perhaps mammalian cells, a kinase signaling pathway is believed to sense deficient amino acid levels and activate the autophagic process (reviewed in [8,9]). Accordingly, ketone utilization in preference to amino acid catabolism might indirectly control protein degradation by sparing amino acid pools. However, some studies question a necessary relationship between amino acid concentrations and the kinetics of protein degradation under all conditions (reviewed in [10,11]). Several reports suggest initially increased muscle protein degradation during early starvation, followed by secondarily decreased degradation under continuing amino acid deficiency of later starvation [2,4,5,12].

Convergent findings now suggest a compelling hypothesis for biphasic pacing of proteome turnover under early and later starvation. Maintenance of most cell protein degradation requires reducing energy [13–17]. Diamide is a pro-oxidative catalyst that promotes the reversible formation of GSSG, protein *S*-glutathione heterodisulfides, and other reactions [18–20]. Diamide reversibly inhibits 75% of total myocardial protein degradation without ATP depletion or contractile dysfunction; however, 25% is redox-independent [15]. We suggested that diamide anti-proteolytic action reveals a metabolic reducing requirement providing integrated catabolic control via multiple effectors including cysteine proteases [13]. Diverse, redox-responsive catabolic mechanisms have now been characterized (reviewed in [16]). Recent proteomic studies speculate that redox-sensitive sulfur sites comprise a coordinate control system, integrating diverse protein functions with the shared redox network [21–26]. A subset of the proteome has been defined as several hundred proteins with sulfhydryls accessible to sulfhydryl-reactive agents. Diamide catalyzes reversible S-glutathionylation of many proteins of the redox-responsive proteome subset. Proteins with redox-reactive sulfur are functionally diverse and include many involved in various aspects of protein turnover (see Discussion). Under constitutive metabolic conditions, reduced proteins might function independently without common controls. However, an increasingly oxidative environment might recruit diverse proteins into pre-emptive, coordinate control secondary to depletion of reductive chains, change of GSH and/or GSSG/GSH status, and perhaps direct redox actions on effector machinery [27–29]. The concept of "disulfide proteome" has been proposed [26]; however, links between protein function and the redox network presumably involve many factors interacting with thiolation–dethiolation reactions.

No measurable definition of oxidative stress is unanimous; however, decreased GSH, increased GSSG/GSH, and ketonemia are associated with an oxidative imbalance after several days of starvation [30–32] or in diabetic ketonemia [4,33–35]. In vivo protein S-glutathionylation has been reported in relation to diabetic oxidative imbalance and ketonemia [36–38]. S-glutathionylated proteins are under investigation as diagnostic indicators of the oxidative status of diabetic ketonemia. Keto oxygens are directly quite reactive with protein sulfhydryls, independent of GSSG formation, and can oxidatively inactivate sulfhydryl enzymes [14]. Indeed, acetoacetate (AA)[1] has long been known as a direct sulfhydryl oxidant at less than physiologic concentrations [39].

Continuing reports reveal that much catabolic machinery is redox-responsive, e.g., ubiquitin conjugation enzymes, the proteasome, overall cytokinesis, etc. (see Discussion). However, cysteine proteases are fundamental models to explore the linkage of proteolysis to metabolism of starvation. Much of cell protein degradation involves cysteine proteases. Despite greatly differing functions and controls, cathepsins, calpains, caspases, and the recently discovered autophagins [40] all share the namesake catalytic cysteine. In addition to heterodisulfide formation, many oxidative reactions of sulfur can inactivate mature cysteine proteases following cleavage of pro-regions (see Discussion). Cysteine protease reaction rates depend upon the concentrations, redox ratios, and reductive potentials of various passive redox influences interacting with active enzyme chains [17]. Keto or quinone oxygens are among multiple non-specific reactants that can participate in oxidative inactivation of cysteine proteases [14]. In contrast to cysteine proteases, some other types of proteases require no reductive activator and are not inhibited by

[1] *Abbreviations used:* CDNB, 1-chloro-2,4-dinitrobenzene, AA, acetoacetate, BHB, 3-β-hydroxybutyrate.

sulfhydryl-reactive agents, e.g., the intracellular metallo-endoproteases and metallo-exopeptidases, and others.

Improved techniques of myocardial perfusion now permit reliable bioassay of proteolytic pathways in a primary tissue under controlled nutrient exposure [16]. Present studies hypothesized an interplay between tissue GSH-GSSG/GSH status and ketone bodies in coordinate, pre-emptive control of protein degradation during later starvation. First, the ex vivo reaction rate of a model cysteine protease is responsive to both GSH and GSSG/GSH status, independent of ketones. Second, in perfused primary mammalian tissue, much of protein degradation is responsive to diamide and other experimental interventions in tissue sulfhydryl status. Third, extra-cellular ketonemia inhibits redox-dependent myocardial protein degradation under constant physiologic amino acid concentrations, suggesting interplay between ketones and GSH-GSSG/GSH in modulating proteolysis.

## Methods

### *Perfusion of hearts*

Small hearts from young 160–180 g rats were perfused as Langendorff preparations as previously described [15,16]. A constant flow rate of 6.8 ml/min provided a low perfusion pressure of 40 mm Hg, without edema and high internal tissue pressure. The non-recirculating perfusate contained Krebs salts, physiologic concentrations of complete amino acids, citrate, pyruvate, lactate, 10 mM glucose, and 0.3% bovine serum albumin, 35 °C (95% oxygen and 5% carbon dioxide). The pH was monitored continuously. All infusates were adjusted to pH 7.4. Perfusate was filtered in-line. These improved conditions provide good contractile function for more than 6 h. This preparation is not stable under other conditions routinely employed historically. For example, mere omission of perfusate albumin causes functional decrement beginning at 10 min. Separate studies reveal that longer term survival can be enhanced with hormonal supplementation; however; such endocrine agents were inconsequential over the present observation period. Experimental agents were infused from 200-fold concentrated solutions into the flowing perfusate or can be added to the reservoir bottle if stable. An infusion immediately above the 1 cc heart at a non-recirculating flow of 6.8 ml/min proceeds through the organ within 10 s of infusion; therefore, reduced GSH infusate does not appreciably oxidize prior to exposure.

### *Bioassay of myocardial protein degradation*

Hearts were biosynthetically labeled ex vivo with [4,5-$^3$H]leucine for 15 min prior to addition of non-radioactive leucine chase to the perfusate (0.15 or 1 mM, as indicated). The release of trichloroacetic acid soluble radiolabel was measured at 2 min intervals from 3 to 5 h post-labeling by collection of the total non-recirculating effluent perfusate with a fraction collector. Muscle can metabolize leucine; however, under present conditions the percent per min metabolism was rigorously determined to be a negligible fraction of the radioactivity released (reviewed in [16]). TCA-soluble postcursor released from this preparation was determined to consist almost entirely of leucine as a single amino acid. Results are qualitatively identical when large amounts of [$^3$H]leucine are injected into the rat 4 h prior to perfusion.

The progress of declining macromolecular [$^3$H]leucine release remaining in the preparation (integral degradation) has been compared to the minute-to-minute rate of [$^3$H]leucine release (differential of degradation) over 20 min–5 h post-labeling. A two-component equation describing the progress of macromolecular [$^3$H]leucine remaining in myocardial protein over 5 h has been previously described [13]. Under constant intracellular conditions, the rate of total [$^3$H]leucine release from 3 to 5 h post-labeling declines continually in proportion to the progressively declining amount of undegraded labeled proteins remaining. The declining control baseline of [$^3$H]leucine release represents the total degradation of diverse proteins with heterogeneous half-lives. Control values of protein degradation are illustrated as the normalized, constant 100% value of the declining baseline of [$^3$H]leucine release. The statistically fitted baselines of [$^3$H]leucine release 45 min prior to inhibition were accurately extrapolated over the following periods of inhibition as verified in uninhibited controls [16]. Solvent or counter-ion controls for all infusates were rigorously characterized. Acetoacetate is supplied as the lithium salt. In the perfused rat heart, lithium has been reported to cause no effect on function at therapeutic 1 mM or supra-therapeutic 10 mM concentrations (extensively reviewed in [42]). In this preparation, infusion of 5 mM lithium, or more, caused no change in contractile function or protein degradation, i.e., less than the experimental uncertainty of $\pm 3\%$.

### *Technical effects of perfusate amino acid concentrations on protein degradation*

Unless reincorporation is prevented, a large experimental increase in protein synthesis might divert a fraction of radiolabeled postcursor from extra-cellular release to reincorporation. Following radiolabeling without extra-cellular leucine, postcursor reincorporation can be prevented either by competitive inhibition with non-radioactive perfusate leucine chase, or elimination of protein synthesis with cycloheximide under physiologic leucine. In the present preparation, these two different methods provided identical conclusions

over 1 h exposures [16]. However, cycloheximide proved toxic after 1 h and could not be routinely employed. In some past experiments, hearts were labeled in the absence of non-radioactive extra-cellular leucine, followed by 1 mM supra-physiologic leucine chase to ensure against unknown changes in postcursor reincorporation caused by adrenergic-induced increases in protein synthesis. However, by sequentially increasing the leucine concentration after labeling it was determined that physiologic 0.15 mM leucine chase suffices to promote at least 96–98% of the release that is observed at maximally effective 1 or 2 mM leucine. As shown statistically below, results with present experimental agents are indistinguishable when physiologic 0.15 mM leucine chase is employed in place of supra-physiologic 1 mM chase. Using cycloheximide (20 μM) to prevent reincorporation, a 1 h perfusion in the absence of any added amino acids resulted in a slight elevation of approximately 5–10% above the basal degradative rate observed with complete physiologic amino acid concentrations (data not shown). This slight elevation in protein degradation upon complete amino acid deprivation is much smaller than the experimental changes of interest. Moreover, the relevance of perfusion with no amino acids is questionable in view of the following decrement in contractile function. Perfusion with 0.5 or 1.5 times the routinely employed physiologic amino acid concentrations caused no observable difference in major results shown below (data not shown).

*Culture and measurement of protein degradation in 3T3 cells*

A31-3T3 cells were cultured as previously described in detail [41]. Cells were uniformly labeled by growth in medium containing [$^3$H]leucine. Non-radioactive cultures were grown in parallel in order to employ the identical depleted replacement medium to measure protein degradation in quiescent cultures. At the time of measurement, cultures were quiescent at a slightly subconfluent density of $2 \times 10^6$ per 5 cm dish, due to depletion of limiting growth factors from unchanged Dulbecco's enriched medium with 6% fetal calf serum. Under these conditions, cultures are not acidified and retain sufficient nutrients to support continued growth upon addition of more serum to unchanged medium. At the time of measurement, radioactive cultures were rinsed and non-radioactive deleted medium from identically grown cultures was employed as replacement media throughout (1 mM non-radioactive leucine). At 3 h, the medium was again replaced without the shock of rinsing in order to eliminate the rapid turnover subcomponent from the measurements as described below. Initial background radioactivity was determined in zero time blanks at 3 h. The percent per hour release of amino acid from culture protein was measured from 3 to 4 h post-labeling as described below. Following the 1 h degradation period, the monolayer was dissolved in 0.1 N NaOH and hydrolyzed prior to determination of percent of radio-labeled monolayer protein converted to TCA-soluble amino acid as described [41]. Protein hydrolysate and medium were neutralized and radioactivity counts were quench corrected. Results differ under injurious conditions of acidified or crowded cultures, or under unchanged minimal essential medium with depleted nutrients, as described [41].

*Protease assay*

Bovine spleen cathepsin B (Sigma) was assayed by standard methods using saturating concentration of *N*-carbobenzyloxy-Phe-Arg-7-amino-4-methylcoumarin (Bachem), (25 μM, 50 mM acetate buffer, pH 5.5), as previously described [17]. Activity was measured fluorimetrically with an automated microwell plate reader equipped with the Deltasoft reaction kinetics program. Reaction rates were statistically fitted within the linear range. Quench of fluorescence was monitored. Protease assay could not be undertaken with 100 μM diamide due to quench of assay fluorescence.

*Reagents*

GSH was freshly dissolved at the time of use. Experimental agents (Sigma–Aldrich), the perfusion apparatus, and other materials were as previously described [15].

## Results

*Steady state cathepsin B reaction rate is graded in relation to two distinct factors: (a) activation by the endogenous range of GSH activator and (b) inhibition by increase above the endogenous GSSG/GSH redox ratio*

The GSH pool concentration and the GSSG/GSH redox ratio are two different variables with potentially different effects on protein functions. The total glutathione pool concentration is determined by the relative rates of synthesis from constituent amino acids vs. loss [31,32]. The GSH/GSSG redox ratio is separately determined by reduction relative to oxidation [43–45]. The constitutive muscle GSH concentration is 2–3 mM with 0.5% GSSG; however, the concentrations and fraction oxidized are believed to differ in various cell compartments [45].

Steady state cathepsin B reaction rate is graded in relation to the endogenous concentration range of GSH activator, and inhibited by increase above the endogenous GSSG/GSH redox ratio (Fig. 1); therefore, the combined inhibitory effects of simultaneous decrease in the former

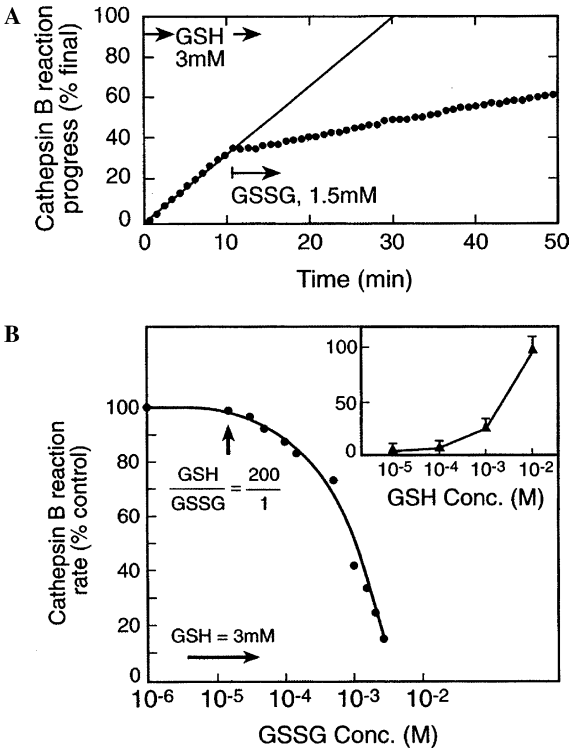


Fig. 1. (A) Influence of GSSG on the progress of cathepsin B reaction previously activated by GSH. Cathepsin B was activated to a constant rate (100% slope in arbitrary units) with 3 mM GSH 5 min prior to the period shown as previously described [17]. At the arrow oxidized GSSG (1.5 mM) was added with rigorous mixing. The immediate, steady state inhibition of the ongoing reaction exhibited the concentration-dependence shown in (B). (B) Dependence of cathepsin B reaction rate on the GSSG/GSH concentration ratio. Cathepsin B was activated to a constant rate with 3 mM GSH for 5 min. GSSG was added at the indicated concentrations for 5 min. The reactions were then initiated by substrate addition and the indicated rates were measured from 5 to 15 min, during which time rates were constant as shown in (A). The dependence of steady state cathepsin B reaction rate upon GSH concentration, without GSSG, is shown ±SD in the inset.

and increase in the latter would be multiplicative. In the absence of GSSG, steady state cathepsin B activity is graded in proportion to the reduced GSH concentration over the endogenous range of 1–10 mM and higher (Fig. 1, and previously described in [17]). Following reductive activation with 3 mM GSH, the addition of 1.5 mM GSSG (50% ratio of oxidized/reduced) promptly caused 78% inhibition of cathepsin B (Fig. 1A). Under some conditions, the protein S-glutathionylating reaction mechanism is reported to involve oxygen-sulfide species, although the exact present reaction mechanisms are uncertain (reviewed in [44,45]). Addition of a series of GSSG concentrations to a constant amount of GSH (3 mM) caused a concentration-dependent inhibition of cathepsin B with inhibitory threshold poised exactly at the constitutive endogenous ratio of 0.5% oxidized/99.5% reduced (Fig. 1B) (however, see Discussion of preliminary results on L-type cathepsins). This threshold inhibitory GSSG/GSH ratio of 0.5% was similar at 2 and 4 mM GSH, although not rigorously characterized. At a ratio of 100 μM GSSG/3 mM GSH (3% oxidized) cathepsin B was inhibited by approximately 15%. A concentration ratio of 200 μM GSSG/3 mM GSH (6% oxidized) would cause 20% inhibition of the intracellular reaction rate of cathepsin B. The 50% effective GSSG inhibitory concentration was approximately 1 mM under 3 mM GSH activator, i.e., 33% oxidized/reduced ratio (Fig. 1). If a fixed initial amount of GSH were increasingly oxidized, the combined loss of 2 GSH with production of 1 GSSG would cause a much steeper GSSG concentration dependence than that shown under addition of increasing GSSG concentration to a constant amount of 3 mM GSH activator.

*Much of protein degradation in myocardium and cultured cells can be inhibited with glutathionylating catalyst (diamide), or sulfhydryl alkylating agent (n-ethyl-maleimide) or glutathione transferase substrate (1-chloro-2,4-dinitrobenzene)*

The redox-dependent subcomponents are defined as the proteolysis that is inhibited by the glutathionylating catalyst, diamide, or other sulfhydryl-reactive agents, or the glutathione transferase substrate, CDNB. The proteolysis that is not inhibited by these agents is defined as redox-independent. Diamide forms a transient intermediate with protein sulfhydryl and GSH so as to catalyze reversible protein-S-SG heterodisulfide formation without permanent diamide–protein adducts [19]. Diamide also catalyzes GSSG formation. NEM forms irreversible, covalent adducts with all accessible protein-SH and depletes GSH. CDNB is an excellent substrate for GSH transferases. Glutathione transferases catalytically form irreversible CDNB adducts with GSH, thereby depleting cell GSH pools presumably without attacking other protein sulfhydryls or causing protein S-glutathionylation.

The same four experimental subcomponents of total cell protein degradation can be distinguished in cultured 3T3 cells and perfused myocardium, although the exact distributions differ quantitatively ([15,41] and Figs. 2 and 3). The [$^3$H]leucine released over the first 3 h post-labeling consists largely of the rapidly degraded subcomponent [46]. The well-known rapid turnover proteolysis consists of approximately 5% of total cell protein that is degraded by 3 h following removal of labeled medium. Although details are uncertain, some of the rapid turnover proteolysis is believed to consist of mis-folded proteins eliminated by a quality control system ultimately involving the proteasome ([46] and see Discussion). Rapid turnover proteolysis is diamide-inhibitable [15]. In present experiments, rapid turnover proteolysis was eliminated from myocardium and cultured cells during a 3 h preliminary degradation period prior to the observation period.

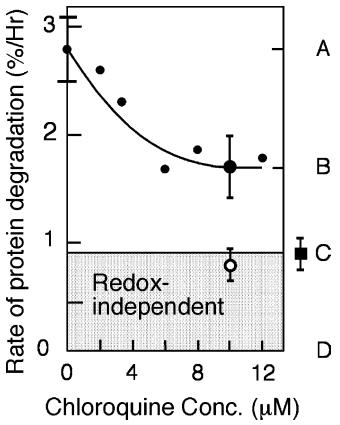


Fig. 2. Three subcomponents of quiescent 3T3 cell protein degradation following elimination of the rapidly degraded subcomponent. A single batch of 3T3 cell cultures was uniformly labeled by prior growth in [$^3$H]leucine (see Methods). Cells were quiescent at a slightly subconfluent density due to selective depletion of limiting serum growth factors from unchanged Dulbecco's medium (6% serum). Reincorporation of radiolabeled postcursor was competitively inhibited by 1 mM non-radioactive leucine chase. Rapidly degraded proteins were eliminated during a 3 h preliminary period prior to the observations beginning at zero time. Results are means (±SD) of five replicate cultures at each point, from a single batch with the same 100% control rate of 2.8%/h. The action of *N*-ethyl maleimide (50 μM) alone (square at right) and 50 μM NEM combined with 10 μM chloroquine (open circle) is shown at single points and insignificantly different ($P > 0.1$). The lysosomal or chloroquine-sensitive subcomponent is A–B. The *n*-ethyl maleimide (NEM)-sensitive or diamide-sensitive subcomponent is A–C. The NEM and diamide-uninhibitable subcomponent is C–D (shaded). The extra-lysosomal, redox-sensitive subcomponent is B–C.

In view of uncertainties as to the basal status of experimental proteolytic subcomponents, their responsiveness to defining agents was compared in uniformly labeled, quiescent 3T3 cells and partially labeled myocardium. In quiescent 3T3 cells, the average rate of total protein degradation is 2.8–3.0%/h following elimination of rapid turnover proteins under conditions specified (Fig. 2 and see Methods). The degradative rate measured by the technique of Fig. 2 has been verified by measurement of the macromolecular leucine remaining in the monolayer over 3–24 h post-labeling [41]. Supratherapeutic chloroquine (10 μM) maximally inhibits and defines the lysosomal subcomponent, consisting of approximately 40% of quiescent 3T3 cell proteolysis (Fig. 2). Chloroquine (10 μM) action required approximately 0.5 h to reach maximal value (data not shown). NEM caused 68% inhibition of the 2.8%/h degradative rate in cultured cells (Fig. 2). Combined exposure to maximal chloroquine (10 μM) and NEM (50 μM) did not cause additive inhibition greater than the action of sulfhydryl agents alone. Therefore, the 40% lysosomal subcomponent is contained within the 68% redox-dependent proteolysis. An extra-lysosomal, redox-dependent subcomponent of approximately 28% is defined by subtraction of the 40% inhibitory action of chloroquine (Fig. 2A–B) from the 68% action of NEM (A–C). The extra-lysosomal proteolytic subcomponents are presumably distributed throughout the cell. In comparison with perfused myocardium (Fig. 3 and below), higher diamide concentrations of 0.5–1 mM were required to cause maximal inhibition in cultured cells (Fig. 2), presumably due to reaction with components of serum-supplemented medium (data not shown). Protein degradation can be highly variable in relation to cell culture conditions as previously described [41]. Essentially all of the proteolysis that varies in relation to culture conditions consists of redox-responsive proteolysis (Fig. 2A–C, NEM-inhibitable), whereas redox-independent proteolysis remains nearly constant (Fig. 2C–D, NEM uninhibitable) (data not shown). Redox-dependent and -independent subcomponents are stable indefinitely under exposures to sulfhydryl-reactive agents at these and higher concentrations (Fig. 3); however, it is not certain that all cell compartments are permeated uniformly.

At 3 h following a brief labeling period, the average rate of myocardial protein degradation (100% control in Fig. 3) is approximately 3.5%/h in association with incomplete representation of slower turnover proteins. Despite predictable differences in basal fractional degradative rates, protein degradation in uniformly labeled 3T3 cells (2.8%/h) and partially labeled myocardium (3.5%/h) was similarly inhibited by maximal concentrations of defining agents (compare Fig. 2 and Fig. 3). In myocardium, chloroquine caused approximately 45–48% inhibition of total protein degradation, as compared to 40% inhibition in 3T3 cells. In myocardium, maximal diamide or NEM caused 76% inhibition as compared to 68% inhibition in 3T3 cells. Diamide/NEM-insensitive proteolysis was 24% of total proteolysis in myocardium and 32% in 3T3 cells (SD/mean of approximately ±12%, or less, in measurements of Figs. 2 and 3). After 3 h of preliminary myocardial perfusion without serum, the lysosomal pathway appears to be stable and fully activated as similarly observed at any time over 3–5 h; however, further activation cannot be ruled out under other conditions.

### *Following diamide washout, reversal of the anti-proteolytic action can be enhanced by artificial repletion of the intracellular glutathione pool*

Kinetics of formation and reversal of protein *S*-glutathione adducts have been described in cardiac myocytes under 0.5 mM diamide exposure and washout [18,19]. When diamide was continuously infused into non-recirculating perfusate, immediately above the heart, a concentration of 100 μM sufficed to cause 95% of its maximal inhibitory action in approximately 1 h (Fig. 3). Following a 20 min exposure to diamide concentrations of 100 μM, reversal of the anti-proteolytic action begins within minutes of discontinuation. The cardiac myocyte has an export mechanism by which

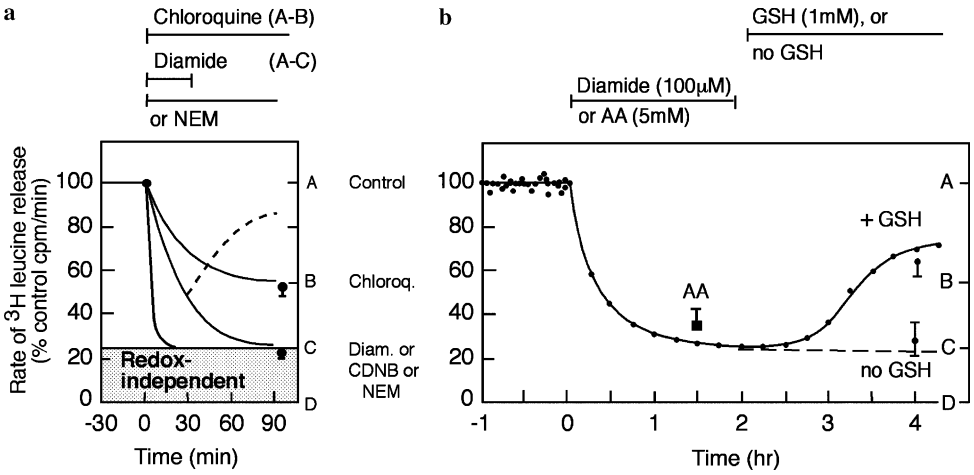


Fig. 3. (a) Redox-dependent and redox-independent subcomponents of protein degradation in the perfused myocardial bioassay as defined by experimental interventions: review. All data shown schematically have been previously described statistically in several reports [13–17]. Zero time was preceded by a 3 h preliminary degradation period to eliminate the rapidly degraded subcomponent. Redox-dependent proteolysis (A–C) is similarly inhibited by diamide (Diam.), 1-chloro, 2-4 dinitrobenzene (CDNB) or *n*-ethyl-maleimide (NEM). The lysosomal pathway is defined as that 45% of total proteolysis that is inhibited by chloroquine (Chloroq.) (A–B). The combined actions of chloroquine and the sulfhydryl-reactive agents are not greater than the actions of sulfhydryl agents alone; therefore, the lysosomal pathway is included in the redox-dependent subcomponents [16]. The action of diamide is largely reversible when discontinued after 20 min exposure (dashed trace; however, see 2 h exposure in (b)). Actions of CDNB or NEM are not reversible over this observation period. The schematic review represents previously described experiments where 1 mM supra-physiologic leucine chase was routinely employed to prevent reincorporation of radiolabeled postcursor [16]. The inhibitions shown ±SD are present experiments where physiologic 0.15 mM nonradioactive leucine chase was employed. Results using physiologic and supra-physiologic leucine were identical (see (b)). (b.1) Reversal of proteolytic inhibition following prolonged diamide exposure with subsequent exposure to glutathione. The rate of [$^3$H]leucine release (cpm/min) from biosynthetically labeled proteins was determined at 2 min intervals as illustrated over the control period 1 h prior to zero time. Single values of the trace fitted from 15 measurements are shown at 30 min intervals. All experiments of (b) were performed with physiologic perfusate concentrations of complete amino acids, including 0.15 mM non-radioactive leucine chase (corresponding to means ± SD in (a)). In contrast to the reversibility following brief diamide exposure (see (a)), the proteolytic inhibition following prolonged 2 h diamide is essentially irreversible in association with presumptive export of oxidized GSSG (dashed trace ±SD and see text). Exposure to supra-physiologic, extra-cellular GSH (1 mM) following otherwise irreversible diamide inhibition caused a partial reversal after a lag of approximately 30 min. (b.2) Inhibition of redox-dependent protein degradation with acetoacetate. Acetoacetate (5 mM) caused a maximal inhibition similar to diamide. Subsequent infusion of diamide following acetoacetate in the identical preparations caused no greater inhibition than the action of diamide alone (data not shown).

oxidized GSSG heterodisulfide or GSH conjugates can be released [47]. Reduced intra- and extra-cellular GSH is also exchanged as observed in skeletal muscle and other cell types [48,49]. Following sufficient conjugation, export of GS–CDNB adducts depletes GSH pools until resynthesized de novo from constituent amino acids. In perfused heart, a 1.5 h exposure to excess GSH oxidants or conjugating substrates is known to deplete most of the tissue content of GSH by export of GSSG or its conjugates [47]. If diamide infusion (100 μM) into the present preparation is discontinued after a brief exposure of 20 min, the incomplete inhibition of proteolysis can be reversed spontaneously to approximately 85–90% of the prior control rate, but not to 100% [15] and schematically reviewed in Fig. 2A. Following an exposure period of 1 h, diamide action is partially reversible to approximately 40–50% of control [15]. However, if diamide exposure is prolonged to 2 h, then the proteolytic inhibitory action is nearly irreversible (Fig. 2B) presumably due to export of the intracellular GSSG pool faster than resynthesis.

Stoichiometric excess of simultaneously infused DTT can reverse the proteolytic inhibitory action of diamide [17]. However, some of this antagonism could be attributable to direct chemical reaction in the extra- or intra-cellular space independent of biologic targets. A role of GSH in maintenance of constitutive proteolysis would be supported if sustained proteolytic inhibition following diamide washout could be restored by repletion of exported GSH. Artificially elevated extra-cellular GSH can be taken up by myocardium and other cell types, and can increase the content of intracellular GSH [50,51]. Therefore, continuous supra-physiologic GSH exposure partially repletes at least part of the exported GSH following diamide exposure. Artificial repletion by grossly supra-physiologic GSH does not simulate in vivo GSH transport or metabolism. It is not certain whether GSH uptake occurs by fluid pinocytosis or some other mechanism or both. Although the supra-physiologic extra-cellular GSH concentration (1 mM) of Fig. 3 is artificial, it is less than the constitutive intracellular myocardial concentration of 2–3 mM. Following prolonged diamide exposure, the otherwise irreversible proteolytic inhibition was largely reversed by artificial repletion of intracellular GSH pools. Therefore, GSH is either involved in supporting the proteolysis decreased

by diamide action or can largely substitute for other factors involved. The individual proteolytic subcomponents involved in reversal were not characterized; however, the extent of reversal by GSH suggests that both lysosomal and extra-lysosomal proteolysis are involved. Compartmental GSH permeation under extra-cellular GSH exposure is uncertain.

*Redox-responsive myocardial protein degradation is inhibited by extra-cellular exposure to ketone bodies*

Acetoacetate produced from acetyl Co A can be reversibly converted to 3-β-hydroxybutyrate (BHB) or irreversibly converted to acetone [4]. AA and BHB are the major ketone bodies while acetone is present in lower concentrations. The BHB/AA concentration ratio is normally near 1; although this can change during starvation. BHB and AA concentrations below 0.5 mM are considered non-ketonemic. Slight elevation is defined arbitrarily above 1 mM and ketonemia is above 3 mM. At 3 days of starvation, ketone concentrations are above 1 mM and after 4 weeks 6–8 mM. In diabetes, 6–10 mM ketones are commonly observed and 20 mM can be attained in severe diabetic keto acidosis.

Infusion of 5 mM AA caused marked inhibition of the same proteolysis that was inhibited by diamide or NEM (Fig. 3b). Because AA and BHB can be interconverted, the ultimate intracellular partition among experimental ketones following AA infusion is not known. Although not rigorously measured, some inhibitory action was clearly observed at 1 mM AA (data not shown). In two experiments, BHB (5 mM) caused at least 60% inhibition; however, the extent of conversion to AA is unknown. Concentrations of 20 μM AA or BHB caused no effect over 1 h (data not shown).

Several independent investigations describe the toxicity of ketone bodies to the perfused heart but not the in vivo heart [6,7]. Present perfusion conditions are not directly comparable; however, a decrement of contractile function was also confirmed in this preparation at 1 h of perfusion with 5 mM AA or BHB, despite maintenance of the pH at 7.4. Acetone at 2 mM was also toxic. These toxicities caution that the perfused heart might exhibit lesser defenses against sudden oxidative challenge than the compensated in vivo organ under gradual transcriptional responses (see Discussion).

Results questioned whether the anti-proteolytic action is specific for the ketone bodies of starvation or rather a general action of keto oxygens. Keto or quinone oxygens are general oxidants of protein sulfhydryls and also inactivate cathepsin B directly [14,39]. A wide variety of endogenous and xenobiotic substances can oxidize protein sulfhydryls with unknown relevance to enzyme redox, including: dehydroascorbic acid, menadione, and others [14,52]. The ring multiketones of dehydroascorbic acid are present at endogenous concentrations below 10 μM, whereas ketones of starvation are present at 1000-fold greater concentrations. The multiketones, alloxan, and dehydroascorbic acid, are potent inhibitors of protein degradation in this myocardial preparation with threshold exposure of approximately 20 μM [14]. Here, it was determined that the effect of acetoacetate (5 mM) was mimicked by menadione (20 μM), although the endogenous relevance of the latter exposure is unknown [52]. The highly reactive, non-biologic oxidant, 1,4-benzoquinone, inhibited all of diamide sensitive proteolysis at 1 h exposure to 10 μM, followed by toxicity (data not shown). Therefore, an anti-proteolytic action can be produced by sufficient concentrations of diverse keto or quinone oxidants other than endogenous ketone bodies; however, potencies can differ markedly. In view of these and other factors, the exact concentration–response of acetoacetate action was not characterized and acetone was not investigated, despite its likely action (see Discussion).

## Discussion

Redox dependency appears to pace lysosomal and some extra-lysosomal proteolysis upon transition from constitutive metabolism to late starvation; however, other catabolism remains simultaneously independent. Many studies have characterized redox-responsive transcriptional regulatory proteins; however, the redox-dependency of bulk protein synthesis has not yet been extensively investigated.

*Interaction of GSH-GSSG/GSH status with ketonemia in regulation of proteolytic pathways*

GSH-GSSG/GSH status and ketonemia appear to be separable, but have interactive influences over protein degradation in progressive starvation. Known shifts in tissue GSH concentration or GSSG/GSH redox ratio might both influence cathepsin B reaction rate in early starvation, prior to ketonemia. In ad libitum fed animals, diurnal fluctuations in tissue GSH concentrations can exceed 20% in association with unknown nutritional and endocrine influences in the absence of GSSG accumulation or ketonemia [30–32]. According to the results of Fig. 1B (inset), the reported 20% diurnal tissue fluctuations in reduced GSH concentration alone could exert an appreciable influence over cathepsin B in the absence of oxidative stress. After several days of starvation, GSH in some tissues can decline by 50% while GSSG can increase by 25%, although reports vary ([31,32], and reviewed in [45]). Thus, cathepsin B reaction rate might decline by more than 50% after only several days of starvation-induced changes in the status of GSH and GSSG/GSH pools (however, see below). In later starvation tissue proteases, are also exposed to

ketones and other interactive oxidative influences in addition to alteration of the sulfhydryl–disulfide redox buffer. Among protein oxidants, the ketone bodies of starvation are neither unique nor extremely potent in inhibiting intracellular protein degradation; however, their very large endogenous concentrations exceed the experimentally effective concentrations by far.

*Large fluctuations in redox-responsive protein degradation without obligatory change of extra-cellular amino acid concentrations*

Amino acid deficiency is proposed to trigger the autophagic process via kinase signaling in yeast, although mammalian correspondence is still uncertain [8,9]. Regardless of linking mechanism, the activation of autophagy might selectively elevate lysosomal protein degradation in early starvation under adequate reducing conditions. However, proteolytic inhibitions can be experimentally induced by ketones under constant concentrations of amino acids found in ad libitum fed animals. Independent of amino acid concentrations, oxidatively inhibited vesicular cysteine proteases would over-ride other controls elevating acquisition of lysosomal substrates. No evidence suggests that amino acids are directly involved in the control of protein-SH/protein-SSG redox equilibrium. Nonetheless, glutathione synthesis in some tissues can be influenced by dietary deficiency of amino acids, particularly cysteine [31,32]. Dietary insufficiency of GSH precursors could be indirect factors in the linkage of protein degradation to nutrition via glutathione synthesis. Moreover, glutamate–cysteine ligase could be an important rate-limiting enzyme in protein degradation as well as GSH synthesis. Inter-organ GSH exchange is appreciable and could be a modifying factor in systemic control of tissue proteolysis [48–51].

*Pre-emptive redox coordination of independent proteolytic controls*

Much of non-lysosomal protein degradation is mediated by calpains, extra-vesicular cysteine cathepsins, perhaps non-apoptotic functions of some caspases, and the ubiquitin–proteasome pathway [16,53–55]. Under adequate reducing conditions, the diverse functions of these distinct catabolic processes might be independently controlled by redox-independent factors. Upon depletion of reductive chains, otherwise independent proteolytic processes might shift to some degree of coordinate limitation. Autophagic proteolysis is dependent upon cysteine proteases via the two mechanisms of lysosomal substrate acquisition [40] as well as intra-vesicular protease function. Speculatively, autophagic activation by the autophagin cysteine proteases might be modified by their oxidative inactivation [40]. In addition to cysteine proteases, other redox-responsive catabolic machinery might also influence proteolytic pathways by diverse roles not fully reviewed here [16,21–28,53]. However, substrate protein disulfide status cannot explain the redox-responsiveness of cathepsin B toward the present synthetic substrate.

In response to gradual onset of oxidative conditions, the in vivo organ might induce proteases, GSH synthesis, redox enzyme chains, and other anti-oxidant defenses that are not rapidly induced in the perfused organ. Opposing anti-oxidative transcriptional responses could partially restore inhibited catabolic functions toward pre-ketonemic levels. Accordingly, uniform exposure to circulating ketones might not cause uniform proteolytic inhibition in all tissues after specialized transcriptional compensation. Present results do not negate oxidative activation of a small amount of proteolysis obscured by the larger amount of oxidative inhibition, or delayed activation of the degradation of oxidized proteins.

*General interactions of ketones and other redox factors with cysteine protease function*

The sulfur reactivity of ketones might directly oxidize a protease if the oxidant collides with the active site before reaction with a competing reductant such as GSH [39]. Second, 5–10 mM keto oxygens might partially deplete lesser concentrations of GSH as passive protease activator while oxidatively producing GSSG as inhibitor. Third, the cyclizing disulfhydryl motif is critical to the function of several reductase chains and is sensitive to oxidants. Therefore, ketones could interfere with the function of reducing chains providing protease activation and/or GSH maintenance. Finally, the oxidative environment associated with 5–10 mM ketones, in the presence of lesser GSH concentration, might non-specifically change the interactions of cysteine proteases with their activator(s) or substrates. Similarly, diamide could inhibit proteases by multiple direct and indirect mechanisms, including accumulation of transient protease-diamide intermediate, alteration in cell GSH, GSSG/GSH status, alteration of substrate protein properties, metal redox status, etc.

In addition to heterodisulfide formation, the sulfur of proteins can react to many products, including sulfenic, sulfinic, and sulfonic oxygenations, nitrosylation, metal liganding, and covalent or non-covalent interactions with many metabolites [14,56,57]. However, the simultaneous distribution of protease sulfhydryls among various oxidation products is not well characterized. The versatility of sulfur suggests that a population of cysteine proteases might exist in a partition among multiple oxidative products, dependent upon conditions in various compartments. Proteolytic controlling roles

of various other redox-active factors remain to be determined.

*Passive and active factors influencing redox-dependent catabolic machinery*

Passive S-glutathionylation–dethionylation equilibria do not comprise a uniform "master switch" over all responsive catabolic mechanisms. According to idealized theory of redox buffering, individual proteins can exhibit unique responsiveness to reduced GSH concentration alone and also the GSSG/GSH redox ratio [1,19,20,29]. Confirmed results from separate studies indicate that the reaction rates of several L-type cathepsins are graded in relation to GSH concentration, but not inhibited by GSSG concentrations that inhibit cathepsin B. Thus, passive redox-responsiveness might differ markedly among various cysteine proteases. Second, under partial oxidation of a population of proteins, the residual submaximal function might be sufficient to serve some roles almost unabated.

Passive thiolation–dethiolation equilibrium of a protein in defined assay does not account for intracellular control by combined passive redox factors and active redox enzyme systems. The intracellular concentration of completely reduced GSH does not passively support the maximal possible function of all proteins requiring reduction, as illustrated with cathepsin B [17]. In theory, the function of some protein sulfur sites might be increased above the function supported by the GSH/GSSG redox buffer by stronger reductants with suitable potentials and reactivities. In addition to passive redox equilibrium, metabolic reductive energy can be actively transferred to proteases by several non-specific enzyme chains, including: NADPH-dependent reductases, thioredoxins, and glutaredoxins [27–29,58] and reviewed in [16]. These three enzyme systems all reduce proteins via the cyclizing vicinal disulfhydryl motif. Dihydrolipoic acid (DHLA) is a permeant, experimental surrogate for the cyclizing disulfhydryl motif of reductase chains. Supra-physiologic DHLA exposure causes an increase above the constitutive rate of cell protein degradation, although the exact relevance to endogenous control is unknown [59]. Individual cysteine proteases could be differentially responsive to overall reductive vs oxidative changes in constitutive redox metabolism.

A large variety of pathogenic conditions are associated with either insufficiency or excess function of various types of cysteine proteases. For example, atherogenic proteolipid accumulation is suggested to result from chronic failure of macromolecular degradation in the diabetic vascular endothelial cell. Keto oxidative inactivation of proteolysis might be relevant to the severe atherogenesis of diabetic ketonemia or other atherogenic conditions [56]. Conversely, apoptosis can be caused by an acute, general hyper-catabolic state involving diverse cysteine proteases in addition to caspases [17]. Nutri-ceutical infusion of ketones might inhibit cysteine proteases in acute hyper-catabolic conditions preceding lethal injury and apoptosis in heart, brain, or other tissues as implied by studies of other topics (e.g. [60–62]).

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