ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 4, 2000
Mary Ann Liebert, Inc.

Review Article

Redox Control of Protein Degradation

THOMAS D. LOCKWOOD

ABSTRACT

This review summarizes evidence that most of cell protein degradation is maintained by pathways transferring energy from glucose to reduction of enzymic and nonenzymic proteins (redox-responsive). In contrast, a major subcomponent of proteolysis is simultaneously independent of the cell redox network (redox-unresponsive). Thus far, direct and indirect redox-responsive proteolytic effector mechanisms characterized by various investigators include: several classes of proteases, some peptide protease inhibitors, substrate conjugation systems, substrate redox and folding status, cytoskeletal-membrane kinesis, metal homeostasis, and others. The present focus involves redox control of sulfhydryl proteases and proteolytic pathways of mammalian muscle; however, other mechanisms, cell types, and species are also surveyed. The diversity of redox-responsive catabolic mechanisms reveals that the machinery of protein turnover evolved with fundamental dependencies upon the cell redox network, as observed in many species. The net redox status of a reversible proteolytic effector mechanism represents the balance between combined oxidative inactivating influences versus reductive activating influences. Similar to other proteins, redox-responsive proteolytic effectors appear to be oxidized by mixed disulfide formation, nitrosation, reactive oxygen species, and associations or reactions with metal ions and various pro-oxidative metabolites. Systems reducing the proteolytic machinery include major redox enzyme chains, such as thioredoxins or glutaredoxins, and perhaps various reductive metabolites, including glutathione and dihydrolipoic acid. Much of mammalian intracellular protein degradation is reversibly responsive to noninjurious experimental intervention in the reductive energy supply–demand balance. Proteolysis is reversibly inhibited by diamide or dehydroascorbic acid; and such antiproteolytic actions are strongly dependent on the cell glucose supply. However, gross redox-responsive proteolysis is not accompanied by ATP depletion or *vice versa*. Redox-responsive proteolysis includes Golgi-endoplasmic reticulum degradation, lysosomal degradation, and some amount of extravesicular degradation, all comprising more than half of total cell proteolysis. Speculatively, redox-dependent proteolysis exhibits features expected of a controlling influence coordinating distinct proteolytic processes under some intracellular conditions. Antiox. Redox Signal. 2, 851–878.

INTRODUCTION

DIVERSE PROTEINS share a common fate in major alternative degradative pathways. Subcomponents of cell proteolysis are incompletely characterized with regard to such features as proteases involved, substrate specificity, subcellular locations, compartmental controls, substrate designation reactions, substrate translocation systems, integration with cell and systemic metabolism, intracell signals, neuroendocrine controls, and roles in cell or intercell function. Recent evidence suggests that most of cell protein degradation requires cell reducing energy; however some proteolysis is redox-independent (Figs. 1–4).

The energy of glucose can be transferred to various cell processes by two major metabolic networks serving distinct cell functions; however, the relationship of proteolysis to either

Department of Pharmacology and Toxicology, School of Medicine, Wright State University, Dayton, OH 45435.

network remains uncertain. The glycolytic pathway and Krebs cycle provide high-energy phosphate bond energy serving such processes as transport, macromolecular synthesis, and mechanical contraction. The pentose phosphate pathway transfers reductive energy from glucose to cell redox chains serving various bioreductive processes. Among bioreductive processes is the reduction of enzymic and nonenzymic proteins and metabolites by various branches of the redox chains. Interconversion pathways provide some degree of metabolic transfer between the two networks; however, the quantitative extent of transfer is limited. The redox network appears to be interactive with the nitric oxide network, suggesting extreme complexity (reviewed in Claiborne *et al.*, 1999; Stubauer *et al.*, 1999).

Over the last several decades, it has become accepted that much of cell protein degradation is controlled by or responds to fluctuations in the cell ATP content (Morgan, 1982; Gronestejski *et al.*, 1985). ATP is not directly involved in bioreductive processes. Some components of the proteasome translocation system exhibit ATPase activity (Suzuki *et al.*, 1997; Baumeister *et al.*, 1998). ATP is also required for several protein conjugation systems serving diverse roles, including a proposed influence on protein degradation (Mizushima *et al.*, 1998). Other ATP requirements for proteolysis include proton or other transport processes, cytoplasmic motility, fluid motion, vesicular-membrane movement, and substrate translocation. In contrast, research in the possible role of the cell reducing network in proteolysis was halted by simultaneous independent reports that protein degradation was unrelated to experimentation believed to reveal the cell redox status (Chua and Kleinhans, 1985; Fagan and Goldberg, 1985). Other reports claimed that some pro-oxidative conditions increased cell proteolysis or caused biphasic effects.

Although not fully reviewed here, many studies of protein degradation are complicated by debatable hypotheses, as well as multiple experimental obstacles. Exposure of cultured cells to metabolic poisons can decrease averaged proteolysis prior to cell death; however, proteolysis is proportional to ATP depletion down to 5% of normal ATP content (Gronestejski *et al.*, 1985). In the absence of mortal injury, ATP levels are normally defended in a narrow range. Severe prelethal ATP depletion can be associated with many co-variables of unknown relevance to normal cell function. Not all processes requiring ATP are controlled by the nonlethal range of ATP fluctuations. In contrast to ATP, the status of the entire redox network or its components can vary markedly as part of normal cell function (Gilbert, 1984; Ziegler, 1985; Prinz *et al.*, 1997; Meister, 1994, 1995; Chen *et al.*, 1995; Wong et al., 1995; Sen and Packer 1996; Zimmer, 1996; Nakamura *et al.*, 1997; Anderson, 1998; Cotgreave and Gerdes, 1998; DiSimplicio et al., 1998; Roy and Packer, 1998; Arrigo, 1999; Atalay and Sen, 1999; Sen, 1999).

We were unable to find support for control of myocardial proteolysis by nonlethal ATP fluctuations (Lockwood, 1985, 1988) and sought a redox dependence (Thorne and Lockwood, 1993; Lockwood, 1997, 1999). The present review surveys evidence suggesting that maintenance of most cell protein degradation requires transfer of energy from glucose to protein reduction. A distinct major proteolytic subcomponent is simultaneously independent of the cell redox network. We refer to this partition of total cell protein degradation as redox-responsive and redox-unresponsive proteolysis.

At the present state of knowledge, this review considers two major questions: Is a large fraction of cell protein degradation maintained by redox metabolism, and what are the likely effector mechanisms? Affirmative answers seem likely; however, a requirement for reductive energy need not imply a control. Therefore, an additional question for continuing research is whether some intracellular protein degradation might be controlled either by fluctuations in the overall status of the redox network or various individual redox-related controls (*e.g.*, Lockwood, 1997; Rossig *et al.*, 1999; Sehajpal *et al.*, 1999; Sen *et al.*, 1999).

CHARACTERIZATION OF INTRACELLULAR PROTEOLYTIC SUBCOMPONENTS WITH EXPERIMENTAL AGENTS, NEUROENDOCRINE FACTORS, AND METABOLIC INTERVENTIONS

Identifiable subcomponents of total cell protein degradation consist of actual localized

compartmental processes, *e.g.*, endoplasmic reticulum (ER)–Golgi proteolysis or lysosomal proteolysis, and experimentally defined responses to inhibitors, activators, neuroendocrine agents, and metabolic interventions. Accordingly, a subcomponent response can consist of an actual degradative control or an interesting experimental result with unknown counterpart *in vivo*. For example, monensin and chloroquine can be employed to inhibit ER–Golgi or lysosomal proteolysis, respectively, thereby identifying actual degradative compartments. However, a permeant nonspecific inhibitor of an entire class of proteases might simultaneously inhibit all enzymes sharing a common active site mechanism regardless of whether these enzymes are normally coordinated under any *in vivo* conditions.

Thus far, four major subcomponents of total cell protein degradation can be experimentally distinguished in perfused rat myocardium (methods described below) or cultured cells by their responsiveness to agents of known action (Fig. 3, and described in Lockwood, 1988, 1999; Thorne and Lockwood, 1993). First, 6% of total cell protein is rapidly degraded in the ER–Golgi compartment with a half-time of approximately 45 min. From 0–2 hr following termination of a biosynthetic labeling period, ^{3}H leucine is released overwhelmingly from the ER–Golgi proteolytic compartment (Fig. 1A,B). After a 3-hr degradative period, the lysosomal inhibitor chloroquine inhibits 45% of remaining proteolysis, thereby defining a second membrane-delineated subcomponent (Fig. 1B,C). Low effective antilysosomal concentrations of chloroquine do not inhibit ER–Golgi degradation. The monovalent cation ionophore monensin inhibits both the ER–Golgi compartment and the lysosomal compartment (not shown). The unique redox probe, diamide, reversibly inhibits 90% of proteolysis at 0–1 hr postlabeling (Fig. 1A–D), and 75% of proteolysis at 3–8 hr postlabeling (Fig. 1B–D); however, a distinct subcomponent of 25% is diamide insensitive (Fig. 1D–E). The pro-oxidative antimalarial drug, primaquine inhibits much of diamide-sensitive proteolysis (Lockwood, 2000). Simultaneous infusion of diamide with chloroquine does not cause additional inhibition exceeding the action of diamide alone. Therefore, the lysosomal process inhibited by chloroquine is included in diamide action. By subtracting the 45% inhibitory action of chloroquine alone (Fig. 1B,C) from the 75% inhibitory action of diamide (Fig. 1B–D), an extralysosomal, redox-dependent subcomponent of 30% is obtained (Fig. 1C,D). This experimental partition of total protein degradation can also be observed in cultured cells, except that the diamide-insensi-

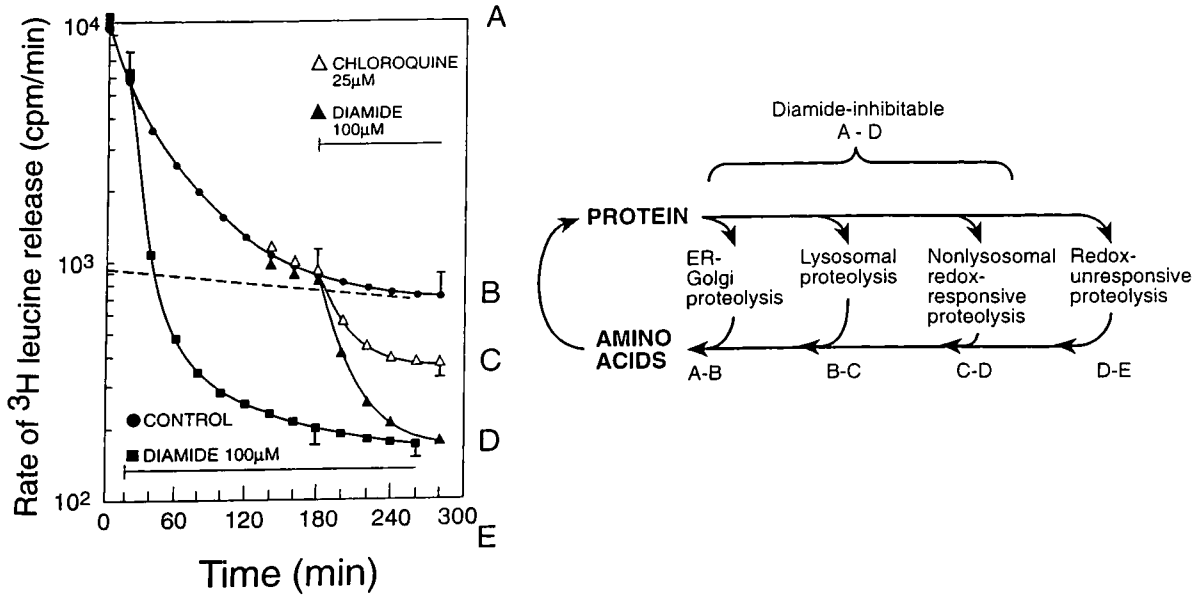


FIG. 1. Experimental partition of total protein degradation into major subcomponents in the isolated perfused rat heart. These four subcomponents can also be found in uniformly labeled cultured cells, except that results are variable in relation to culture condition.

tive subcomponent is 30–35% as compared to 25% in partially labeled myocardium. Much of the diamide-inhibitable subcomponents can also be inhibited by nonspecific inhibitors of sulfhydryl proteases; however, the diamide-resistant subcomponent is also resistant to sulfhydryl protease inhibitors. Interestingly, monensin, chloroquine, primaquine, and sulfhydryl protease active site inhibitors are all effective antiparasitic agents acting by different initial mechanisms (Barret 1997; Coombs and Mottram, 1997; McKerrow, 1999).

Interpretive limitations surrounding the present experimental partition of total cell protein degradation are obvious. The general features of the two membrane-delineated compartments are well known; however, the other two experimental subcomponents are presently identified only by their common responsiveness or nonresponsiveness to experimental interventions. Individual proteolytic controls might violate any generalities observed as the average of major subcomponents. A reductive requirement for most proteolysis need not be irreconcilable with some limited amount of proteolysis that is activated by pro-oxidative conditions, perhaps within the diamide-unresponsive subcomponent.

PROTEIN REDOX REACTIONS AND PATHWAYS TRANSFERRING REDUCTIVE ENERGY FROM GLUCOSE TO CELL PROTEINS

Redox reactions in protein folding and enzyme function

The cytoplasm of mammalian cells and microbes is normally reduced. In the mammalian cell, the redox ratios of NADPH/$NADP^+$, reduced glutathione/oxidized glutathione (GSH/GSSG), and ascorbic acid/dehydroascorbic acid (AA/DHA) are more than 100:1. The cell redox network is involved in both oxidoreductase reactions of protein folding and other types of redox reactions involving protein function and enzyme activities (discussed in Kerblat *et al.*, 1999). The overall reduced state of the cytoplasm interferes with the oxidative formation of intramolecular disulfides during protein folding. In the mammalian ER or bacterial periplasm, some proteins undergo repeated oxidations and reductions before mismatched internal disulfides attain their native disulfide bonds (Bader *et al.*, 1999; Bessette *et al.*, 1999; Debarbieux and Beckwith, 1998, 1999; Rietsch *et al.*, 1997). Although the difference between a nascent unfolded protein and a native disulfide bonded protein involves net oxidation of sulfhydryls to disulfides, protein disulfide oxidoreductase systems repeatedly reduce intermediate oxidized states of the protein during its progress to the native state. Despite interrelationships, it is helpful to distinguish structural oxido-reductions from other areas of protein reduction, such as redox-responsive enzyme activations, which are also mediated by cell reductase chains (Fig. 2). For example, immunoglobulin degradation can be simultaneously influenced by protease activation and substrate reduction (Kerblat *et al.*, 1999).

Relative rates of protein oxidation versus reduction and net protein redox status

The redox state of some proteins represents the net result of their relative rates of oxidation versus reduction (Prinz *et al.*, 1997; Sies 1997; Stewart *et al.*, 1998; Aslund and Beckwith, 1999a,b; Bader *et al.*, 1999; Debarbieux and Beckwith, 1999). Even under the overwhelmingly reduced state of the cytoplasm, proteins such as cathepsin B can undergo a variety of spontaneous oxidative reactions resulting from various metabolites, reactive oxygen species (ROS), nitric oxide (NO), metals, and xenobiotics (Table 1). The cell antioxidant system and reductive system serve to prevent or reverse some types of protein oxidations (Fernando *et al.*, 1992; Jain *et al.*, 1992; Meister, 1992, 1994; Muller, 1996; Slekor *et al.*, 1996; Sies, 1997; Ursini *et al.*, 1997; Luikenhuis *et al.*, 1998; Paget *et al.*, 1998; Hayes and McClellon, 1999; Lee *et al.*, 1999a; Rodriguez *et al.*, 1999). The major reservoir of the antioxidant system, AA, and the major reservoir of the reducing system, GSH, both undergo spontaneous oxidation in the presence of suitable electron acceptors (Rusakow *et al.*, 1995; Anderson, 1998; Banhegyi *et al.*, 1997). Reduced AA and GSH are maintained by transfer of reductive energy from glucose through redox chains to reduc-

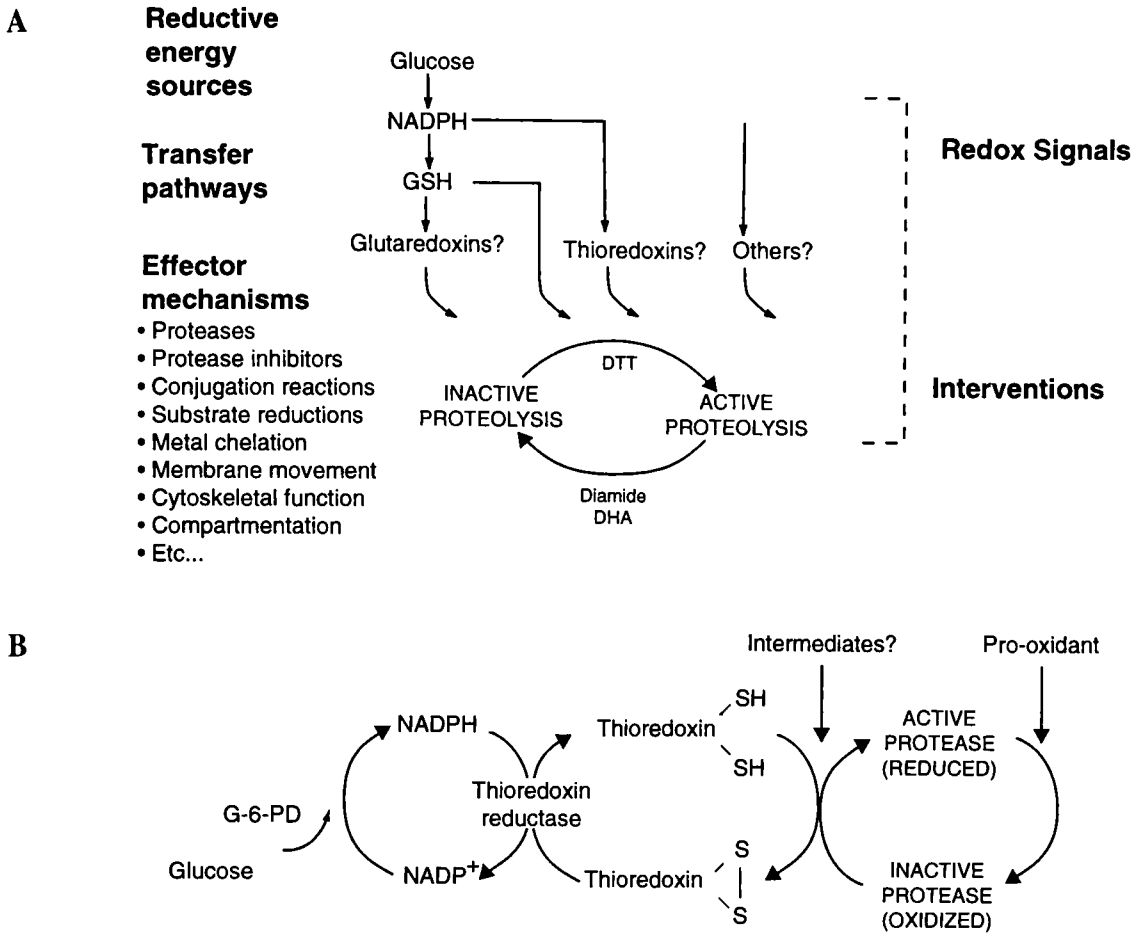


FIG. 2. (A) Selected topics in redox-responsive protein degradation. Enzymes of the redox chains are described in the text. Multiple types of direct or indirect proteolytic effector mechanisms are proteins that are responsive to the intracellular redox network. The degradative machinery can undergo many types of oxidative and reductive reactions; and the net redox status represents the balance between multiple oxidative versus reductive influences. The major reductive chains shown are only two of the conceivable mechanisms by which reductive energy can be transferred from glucose into and out of a protein. A third enzyme system, protein disulfide isomerase, catalyzes repeated reductions and oxidations of nascent protein sulfhydryls during folding and native disulfide formation. Other reductive pathways include lipoic acid which can be reduced by thioredoxin reductase (Fig. 5). Proteins can be reduced by metabolites such as GSH or dihydrolipoic acid or oxidized by metabolites such as DHA, ubiquinone, or menadione and others. Extracellular exposure to diamide or slightly supraphysiologic DHA inhibits intracellular protein degradation; and these actions can be reversed by simultaneous exposure to DTT. DTT can reduce intracellular redox chain components, various pro-oxidative experimental agents, and many oxidized intracellular proteins. The onset and reversal of pro-oxidative antiproteolytic intervention is strongly dependent upon the cell glucose supply (Lockwood, 1999). **(B)** A hypothetical pathway transferring reductive energy from glucose to the activation of a protease. Some sulfhydryl proteases of all phyla undergo spontaneous oxidative inactivation. *In vitro* assay of sulfhydryl proteases is activated by sulfhydryl reductant and metal chelator. Oxidized forms of papain and cathepsin B can be reductively reactivated by the purified NADPH dependent, thioredoxin reductase–thioredoxin system (Stephen *et al.*, 1993; Kerblat *et al.*, 1999). Other possible reductants include lipoic acid (Holmgren, 1979b; Sen *et al.*, 1999) (Fig. 5). These combined findings from separate investigations in conjunction with the glucose dependence of redox-responsive protein degradation (Lockwood, 1999) permit construction of hypothetical pathways from glucose to the reductive activation of protein degradation. GSH can nonenzymatically activate sulfhydryl proteases with much less potency than disulfhydryl agents (Fig. 4).

tion of DHA and GSH disulfide (Bergsten *et al.*, 1990; Wells *et al.*, 1990; Coassin *et al.*, 1991; Martensson and Meister, 1991; Washko *et al.*, 1992; Meister, 1994; May *et al.*, 1995, 1997, 1998; Rusakow *et al.*, 1995; Krauth-Siegel and Ludemann, 1996; Park and Levine, 1996; Foyer and Mullineaux, 1998; Ishikawa *et al.*, 1998; Mendriatta *et al.*, 1998a,b; Washburn and Wells, 1999a,b) (Fig. 4). Reduced GSH can participate in both antioxidant and reductant actions; however, a possible role of AA in reductive processes is uncertain. The same path-

TABLE 1. SELECTED SULFUR REDOX REACTIONS INFLUENCING CATALYTIC AND REMOTE SITES OF PROTEASES AND OTHER ENZYMES

A.	$PrS\text{-}SR_1 + RSH$	$\longrightarrow PrSH + R_1S\text{-}SR_2$
B.	$PrS\text{-}SR_1 + TRX(SH)_2$	$\longrightarrow PrSH + RSH + TRX(S\text{-}S)$
C.	$PrS\text{-}SR + 2GSH$	$\xrightarrow{GRX} PrSH + RSH + GS\text{-}SG$
D.	PrS-OH	
E.	$Pr{\bullet}M^n$	$\xrightarrow{?}$
F.	Pr•Metabolite	
G.	PrS-NO	

Proteins can oxidize to the structures shown at the left, and other structures not shown. The cell antioxidant systems and reductive systems can prevent or reverse some protein oxidations. The functions of some proteins are inactivated by oxidations, *e.g.*, most sulfhydryl proteases, whereas others are activated, *e.g.*, some peptide protease inhibitors. Each individual protein can potentially differ in its tendency to undergo these and other oxidative versus reductive reactions under the identical redox environment. Indeed, some of these reactions might involve specific interactions with metals or metabolites. In A–C, RSH are nonprotein sulfhydryls that can form mixed disulfides with protein sulfhydryls, *e.g.*, cysteine, GSH, or synthetic monosulfhydryls. Reaction A is a disulfide-sulfhydryl exchange of redox state. Reaction B is disulfide reduction by thioredoxin disulfhydryls (see also Fig. 1). Reaction C is disulfide reduction by glutaredoxin-catalyzed GSH oxidation. Reaction C can occur nonenzymatically. Reaction D is reduction of protein oxygenation that can be catalyzed by thioredoxin. The cellular basis for prevention or reversal of a protein–metal interaction (E) is largely unexplained. Some oxidative protein sulfhydryl interactions with metabolites (F) such as DHA are reversible; however, some protein–metabolite conjugations are irreversible, *e.g.*, reactive intermediates of many xenobiotics. Protein–metabolite interactions such as DHLA can also be reductive. Amino acids in addition to cysteine are also redox-responsive, *e.g.*, histidine or methionine. The cell redox network can also interact with protein prosthetic groups such as heme or flavins (not shown). Protein nitrosothiol metabolism (G) and the relation to redox metabolism is controversial (reviewed by Claiborne *et al.*, 1999; Stubauer *et al.*, 1999).

ways reducing GSSG and DHA can also reduce proteins as alternative competing substrates.

Some protein oxidations are irreversible, and some reductions are rather stable. Spontaneous oxidation to protein sulfoxide has been suggested to be reversed enzymatically by the thioredoxin system and perhaps other mechanisms (Claiborne *et al.*, 1999). Protein-mixed disulfide formation is reversible by several reductases (Miller *et al.*, 1991; Holmgren and Aslund, 1995; Holmgren and Borgstedt, 1995; Jung and Thomas, 1996). A familiar reversible protein redox reaction integrated with cell redox function is the transition between Fe^{2+} and Fe^{3+} of hemoglobin (Oliveira *et al.*, 1999) (described below). Cell metal homeostasis and sulfur redox status are intimately related (Cornell and Crivaro, 1972; Sigel *et al.*, 1978; Gromer *et al.*, 1998; Sasoda *et al.*, 1999; Shi *et al.*, 1999). Metal and sulfhydryl redox can be simultaneously influenced by exposure to some of the same exogenous agents, such as antimalarial drugs (Vasquez-Vivar and Augusto, 1992; Fryauff *et al.*, 1995; DiSimplicio *et al.*, 1998). Some other protein oxidations are irreparable and require degradation and resynthesis for restoration of protein structure.

Biologic and pharmacologic reactions of sulfur and vicinal disulfhydryls

Redox reactions of protein sulfhydryls include formation of intra- or intermolecular disulfides, oxygenation products, metal ligands, nitrosothiols, and covalent or noncovalent interactions with a large number of cell metabolites (Table 1) (DiSimplicio *et al.*, 1998). Endogenous metabolic controls associated with reversible oxygenations, nitrosations, and protein-mixed disulfide reactions have been suggested, including proteolytic controls (Gilbert, 1984; Ziegler, 1985; Claiborne *et al.*, 1999; Sies *et al.*, 1999; Stubauer *et al.*, 1999 and see below).

The thioredoxin motif is found in a growing number of thioredoxins, glutaredoxins, and protein disulfide isomerases, and their reaction mechanisms are currently under investigation (Holmgren and Aslund, 1995; Holmgren and Bjornstedt, 1995; Muller *et al.*, 1996; Park and Levine, 1996; Jordon *et al.*, 1997; Zhong *et al.*, 1998; Aslund and Beckwith, 1999b; Ferrari and Soling, 1999; Langenbach and Sottile, 1999; Lee *et al.*, 1999b; Lillig *et al.*, 1999; Mossner *et al.*, 1999; Pedrajas *et al.*, 1999). A similar reversible disulfhydryl-disulfide active site motif is found in the three types of reductases: Cys-X-X-Cys, where X-X is a dipeptide sequence of variable composition in different enzymes. The vicinal disulfhydryls of the thioredoxin motif have been considered to be the endogenous coun-

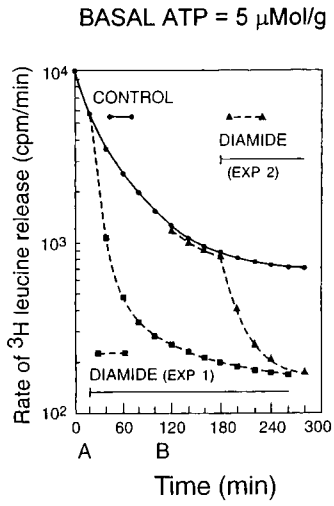
terpart of dithiothreitol (DTT) insofar as the intramolecular oxidation to a disulfide can be coupled to the nonspecific reduction of many types of protein or nonprotein electron acceptors. The disulfhydryls of the thioredoxin motif or dihydrolipoic acid can reduce a large variety of protein disulfides, nonprotein disulfides, oxygenated sulfur, and endogenous metabolites such as ketones, metals, and pharmacologic agents (Yang *et al.*, 1983; Wynn *et al.*, 1995; Holmgren, 1995; Holmgren and Aslund, 1995; Wynn *et al.*, 1995; Arner *et al.*, 1996; Prinz *et al.*, 1997; Huber-Wunderlich and Glockshuber, 1998; Lundstrom-Ljung *et al.*, 1999; Oliveira *et al.*, 1999; Shi *et al.*, 1999). The pharmacology of some useful exogenous redox-active agents bears analogy to endogenous redox reactions and enzyme mechanisms including some degree of nonspecificity. Vicinal intramolecular disulfhydryls of the thioredoxin motif, dihydrolipoic acid, and some synthetic disulfhydryl agents exhibit several chemical properties involved in their actions as effective reductants. The immediate proximity of constrained disulfhydryls in a microenvironment creates the equivalent of a large concentration of unattached monothiols, thereby kinetically enhancing the formation of oxidized internal disulfides when a suitable electron acceptor is available. In Cleland's original paper (Cleland, 1964), it is discussed that the formation of a stable cyclic intramolecular ring disulfide is the key to coupling the internal oxidation of vicinal DTT disulfhydryls to the reduction of other molecules. At least some functions of mutated thioredoxin reductase or GSH reductase can be replaced by exposure of bacteria to synthetic disulfhydryl agents; therefore, disulfhydryl agents seem to be a pharmacologic substitute for some functions of the cell redox network (Prinz *et al.*, 1997; Aslund and Beckwith, 1999b; Debarbieux and Beckwith, 1999). Glutaredoxin draws upon the large reductive reservoir of GSH, whereas thioredoxin utilizes NADPH for protein reduction.

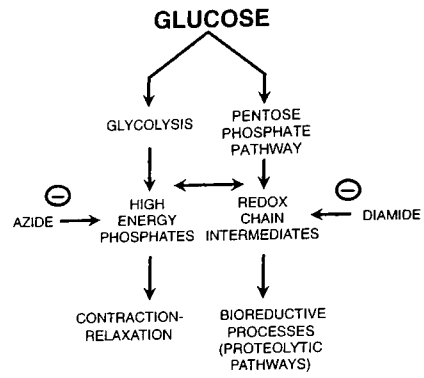
Kosower's reagent diamide is a unique nontoxic agent that can promote the formation of disulfides from monosulfhydryls (Biaglow and Nygaard, 1973; Miller *et al.*, 1991; Kosower and Kosower, 1995; Jung and Thomas, 1996; Dafre and Reischl, 1998). Diamide apparently does not form stable adducts with sulfhydryls, but rather promotes their oxidation via an intermediate mixed sulfide. Nonetheless, some amount of mixed diamide-sulfide intermediate can presumably accumulate under some conditions. Diamide can also react with nonsulfhydryl molecules such as NADPH, although with a much lesser rate. The oxidation of DTT can be directly coupled to the reduction of diamide or thioredoxin or other proteins. Therefore, exposure of cells to stoichiometric excess of DTT concurrent with lesser amounts of prior diamide can simultaneously reduce intracell diamide as well as the reversibly oxidized cell targets of diamide action. In some intracellular experimentation, we have found it informative to impose pro-oxidative conditions with diamide, DHA, or alloxan followed by antagonistic reduction of both exogenous oxidant and oxidized cell targets using simultaneously infused excess DTT under continued exposure to oxidant (Fig. 4).

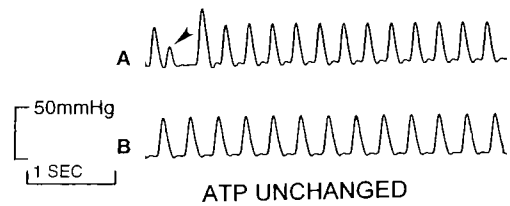
Mono- or disulfhydryls form bonds with a wide variety of biologic and nonbiologic metals (Cornell and Crivaro, 1972; Sigel *et al.*, 1978; Aposhian, 1983). The cell redox network is interactive with metal homeostasis including zinc, copper, and iron (Sigel *et al.*, 1978; Vulpe and Packman, 1995; Reyes, 1996; Gromer *et al.*, 1998; Oliveira *et al.*, 1999; Sasoda *et al.*, 1999; Shi *et al.*, 1999). Indeed, regulation of metals and redox status is hopelessly interrelated for purposes of some types of intracellular experimentation. Vicinal disulfhydryls such as dihydrolipoic acid (DHLA) are simultaneously reductants, and also metal chelators (Sigel *et al.*, 1978). The Zn^{2+}–DTT complex has a stability constant of 10^{11} (Cornell and Crivaro, 1972).

Oxygen and various ROS can oxygenate sulfur to -SOH (sulfenic), $-SO_2H$ (sulfinic), or $-SO_3H$ (sulfonic) states (Claiborne *et al.*, 1999). Reductive reversal of protein sulfenic acids has been reviewed (Claiborne *et al.*, 1999). Sulfoxide is a tautomer of sulfenic acid. Enzymatic reversal of various other sulfhydryl oxygenations and their metabolic roles in mammalian cells is uncertain. Sulfur nitrosylation is a new area in rapid development (Stubauer *et al.*, 1999); and its significance is uncertain (Rossig *et al.*, 1999; Sehajpal *et al.*, 1999).

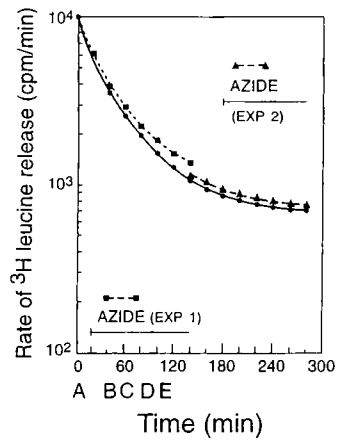
A wide variety of endogenous or exogenous

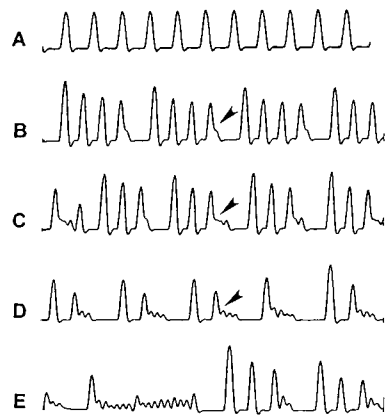




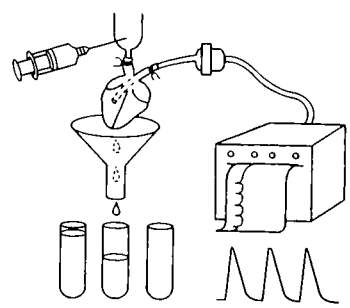


ATP UNCHANGED





ATP DECREASED ~60%



ketones, aldehydes, or epoxides can react with sulfur to form covalent adducts or unrecognized noncovalent associations, as well as promoting disulfide formation. Among the first experimental protein sulfhydryl oxidizing agents was the ring tetraketone alloxan, which is a potent sulfhydryl protease inhibitor (Winterbourne and Munday, 1989; Lenzen and Munday, 1991). Sulfhydryls are believed either to reduce alloxan to dialuric acid or form covalent adducts with its vicinal keto oxygens, probably with multiple coordinations. DHA is an endogenous ring trike-

FIG. 3. **Dependence of a myocardial proteolytic subcomponent on redox metabolism and independence of ATP fluctuations.** The release of incorporated [^{3}H]leucine from proteins was studied in the effluent perfusate of the rat heart under nonrecirculating perfusion (see text and Lockwood, 1999). Ventricular contractile rhythm was characterized simultaneous with release of [^{3}H]leucine postcursor using a fluid-filled ventricular catheter connected to a pressure transducer and recorder. The letters designating contractile rhythms shown at the right correspond to the letters at the indicated perfusion times at the left. ATP was measured in ventricular biopsies. The perfusate contained glucose and physiologic citrate, pyruvate, lactate, complete amino acids, and albumin. These preparations contained 10 m*M* glucose; however, the effects of different glucose concentrations on protein degradation have been described. The initial rate of release of [^{3}H]leucine has been normalized to 100% of 10^4 cpm/min (see Lockwood, 1999). In separate experiments 1 and 2, conclusions were similar whether diamide or azide were infused at 20 min (Exp. 1) or 3 hr (Exp. 2) postlabeling, excluding a 20-min preliminary washout period. Sodium azide can cause a measured 60% decrease in ATP and severe contractile dysrhythmia without accompanying change in protein degradation. The arrows indicate progressive inability of the myocardium to relax under increasing ATP deficiency. Conversely, diamide caused a large inhibition in protein degradation with no accompanying change in tissue ATP content or impairment of contractile function. This action of diamide is metabolically 85% reversible within 1–2 hr of discontinuation. The arrow of the control rhythm indicates a normal premature ventricular contraction typical of the *in vivo* heart.

tone with structural and reactive homology to alloxan, including strong sulfhydryl reactivity and protease inhibition. Ubiquinone, menadione, and DHA have been suggested as endogenous protein oxidants (Banhegyi *et al.*, 1998; Debarbieux and Beckwith, 1998, 1999; Bader *et al.*, 1999). Among endogenous reductants, DHLA is an interesting disulfhydryl with protein and protease reductant properties (Holmgren, 1979a,b; Packer *et al.*, 1995; Arner *et al.*, 1996; Sen *et al.*, 1999) (Fig. 5). Importantly, a redox-active protein might be simultaneously influenced by multiple interactions surveyed above.

Sources of cell reductive energy and transfer to redox chains

NADPH is the primary reductant driving distal reductive processes by maintaining reduced reductases (Zimmer, 1996). In rat heart, multiple enzymes can be involved in the production of NADPH (Satrustegui and Machado, 1980). However the majority of the NADPH requirement is met by oxidation of glucose-6-phosphate via the pentose phosphate pathway.

The pentose phosphate pathway is rate-limited primarily by glucose-6-phosphate dehydrogenase (G-6-PD). The rate of reaction of G-6-PD is strongly influenced by cell levels of NADPH/$NADP^+$, although in various species some other influences have been suggested such as thioredoxin (Gleason, 1996). The evolved maximal capacity of activated G-6-PD to supply cell reductive energy is insufficient under some situations involving increased oxidative demands such as parasitism (Bozzi *et al.*, 1996; Barret, 1997; Krauth-Sigel and Coombs, 1999).

The prevailing rate of reductive energy transfer and net levels of reduced chain intermediates represent a supply–demand balance. The reduced state of redox chain intermediates can be depleted experimentally by either an insufficiency of glucose supply feeding the chains or an excess demand for reductive energy caused by a wide variety of endogenous metabolites or exogenous agents. Upon some types of increased oxidative demand, cell stores of GSH can be oxidized first followed by secondary NADPH depletion as NADPH is drawn to provide GSH repletion via GSH reductase (Biaglow and Nygaard, 1973; Kosower and Kosower, 1995). Despite all remaining unknowns, the cell reductive chain status can be moderately depleted by increased oxidative demand without accompanying ATP depletion or appreciable pathogenesis (Spyrou and Holmgren, 1996; Lockwood, 1999). Nonetheless, severe sustained depletion of cell reducing energy is eventually injurious, thereby recruiting a series of prelethal responses that are not relevant to normal or physiologically stressed cell function.

Redox chains transferring reductive energy from glucose to protein reduction and proteolysis

Oxidized thioredoxin receives reductive energy from NADPH via NADPH-dependent thioredoxin reductase; and reduced thioredoxin transfers reductive energy to a wide variety of oxidized substrates (Fig. 2). Oxidized GSH receives reductive energy from NADPH via GSH reductase. Glutaredoxin catalytically couples the oxidation of GSH to GSSG and reduction of many substrates (Holmgren, 1995;

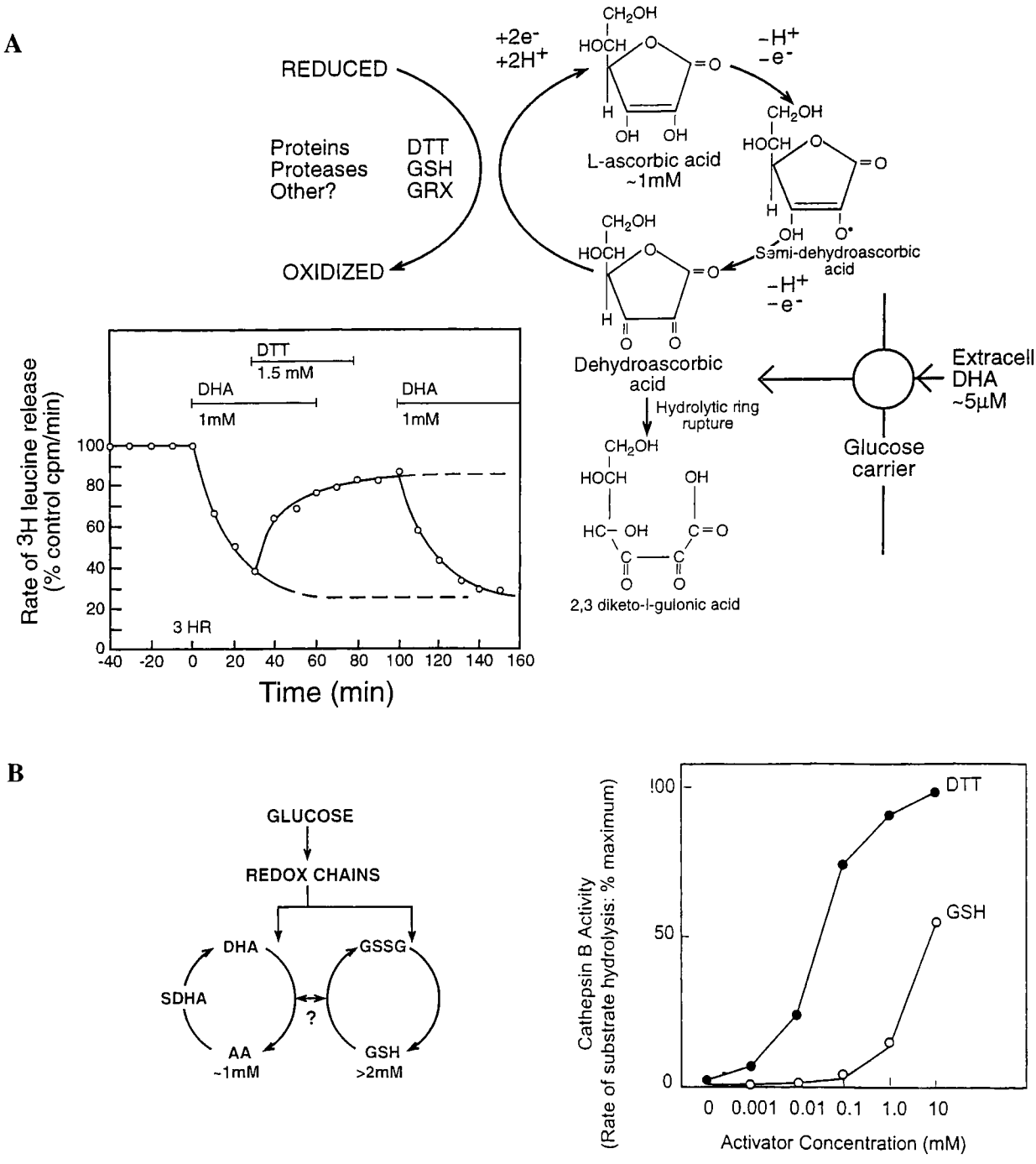


FIG. 4. (A) Effect of supraphysiologic DHA on myocardial protein degradation and reversal by simultaneous DTT. The structures of the AA, SDAA, DHA cycle are illustrated (reviewed in Washko *et al.*, 1992; Banhegyi *et al.*, 1997). In the presence of an electron acceptor, AA spontaneously oxidizes to SDAA and DHA. Cell reductases reduce DHA back to AA. DTT is known to reduce DHA to AA and also reduces oxidized intracellular targets of DHA action. Physiologic extracellular DHA concentrations of 5 μM caused no effect on proteolysis: however, a threshold concentration of only 15 μM caused a gradual onset of proteolytic inhibition over several hours (Lockwood, 1997). The large excess millimolar DHA concentrations shown were employed to achieve the inhibition and reversal within the observation period. Simultaneous infusion of excess DTT largely reversed the action of continued DHA. **(B)** Monosulfhydryl and disulfhydryl activation of cathepsin B. Purified bovine cathepsin B solution was permitted to inactivate spontaneously under air, by approximately 97%. The inactive protease was preincubated for 15 min in the indicated concentrations of DTT or GSH prior to initiation of assay by addition of concentrated substrate. The substrate was the fluorogenic dipeptide derivative, benzyloxycarbonyl-Arg-Arg-7 amido-4-methylcoumarin (25 μM, pH 6.5), assayed by standard methods. The 50% maximal activity occurred at approximately 50–100 μM DTT; however, this amount of activity required approximately 5 mM GSH. It remains unknown whether the AA/DHA or GSH/GSSG cycles might play signaling or controlling roles in linking redox metabolism with some protein degradation. GSH can nonenzymatically reduce DHA.

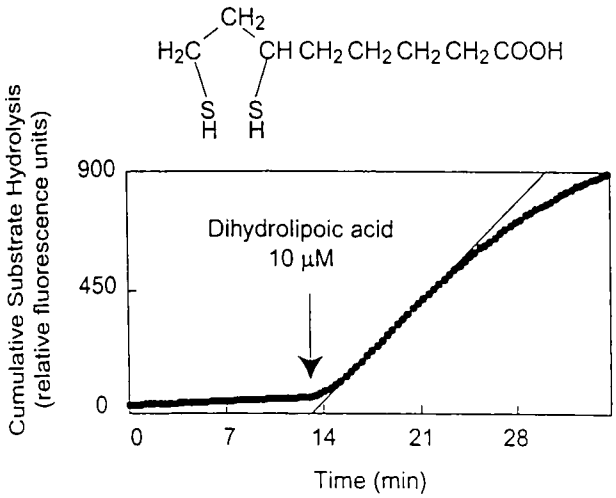


FIG. 5. Activation of cathepsin B by DHLA. Spontaneously inactivated cathepsin B was combined with fluorogenic dipeptide substrate at zero time, revealing little initial activity. At the time indicated by the arrow, a 200-fold concentrated solution of DHLA in dimethylsulfoxide was added to a final concentration of 10 μM. Separate solvent controls were negative. In separate experiments, the action of 1.5 μM DHLA was similar to that illustrated for 10 μM. The maximal rate of reaction is given by the maximal slope of the reaction progress. Deceleration of the reaction is due to depletion of substrate. The maximal reaction rate caused by DHLA at 1–10 μM is similar to the maximal activating action of much higher DTT concentrations near 100 μM, as shown in Fig. 4 with the same substrate.

Holmgren and Aslund, 1995; Yang *et al.*, 1988; Nordstrand *et al.*, 1999). The various types of thioredoxins and glutaredoxins might exhibit some degree of substrate binding specificity; but many different protein and nonprotein targets can be nonspecifically reduced by either of these systems (Aslund and Beckwith, 1999a,b; Bessette *et al.*, 1999). It is suggested that some of the same proteins can be reduced by either glutaredoxin or thioredoxin, thereby providing alternative redundant reductive pathways using either GSH or NADPH. Lipoic acid might be a redox shuttle reduced by thioredoxin reductase (Arner *et al.*, 1996).

REDOX SIGNALS AND CONTROLS AND EXPERIMENTAL INTERVENTION

The unwieldy concept of the redox network involves much of cell metabolism and many reactions in distinct compartments (Claiborne *et al.*, 1999; Stubauer *et al.*, 1999). A moderate pro-oxidative shift is noninjurious and part of normal cell function. Even under a significant oxidative stress, ribonucleotide reductase can continue to reduce a sufficient amount of ribonucleotides to deoxyribonucleotides so as to allow continued cell proliferation (Spyrou and Holmgren, 1996; Cotgreave and Gerdes, 1998). Thus, redox-dependent machinery is not uniformly sensitive to noninjurious oxidative stress, and some proteolysis might be preferentially sensitive (Claiborne *et al.*, 1999).

Beyond the present scope, a very large number of cell systems can be considered to be direct or indirect redox signals, or controls; and many might influence proteolytic effector mechanisms. Major redox signals believed to be "sensed" include sulfhydryl-disulfide status (*e.g.*, Paget *et al.*, 1998; Aslund and Beckwith, 1999a), metal redox status (*e.g.*, Oliveira *et al.*, 1999), various ROS (*e.g.*, Chance *et al.*, 1979; Lee *et al.*, 1999), NO (Stubauer *et al.*, 1999), and many metabolites including lipids, carbohydrates, and peptides. In diverse organisms and cell types, pro-oxidative agents such as metals, ROS, or sulfhydryl oxidants elicit changes in expression of diverse genes revealing the functions, mechanisms, and complexities of the cell redox network (Sen and Packer, 1996; Slekar *et al.*, 1996; Ursini, 1997; Mehta *et al.*, 1998; Paget *et al.*, 1998; Wagle *et al.*, 1998; Arrigo, 1999; Lee *et al.*, 1999b; Salremini *et al.*, 1999). By implication, many redox-responsive genes might indirectly influence proteolytic controls. For example, adrenergic agonists stimulate mechanical function and metabolic demand, and increase the myocardial cell content of G-6-PD. Myocardial G-6-PD induction under external functional demand is consistent with adaption to stress within the redox network. Adrenergic agonists also decrease myocardial protein degradation (Lockwood, 1988).

Supply–demand balance in the cell redox network and oxidative or reductive stresses

The redox system promptly moves to offset increased demand for reducing energy by activating G-6-PD in response to deficiency of reduced intermediates such as NADPH or, perhaps, thioredoxin (Gleason, 1996). Accordingly, a large number of pro-oxidative substances might enter the system at various points so as to increase demand and appear as redox signals. The net overall reductive status of the re-

dox network can be experimentally altered by either a deficiency of glucose supply or increased demand caused by ROS, endogenous metabolites, or xenobiotic substances. Both the total content and rate of reductive energy flow through the system to proteolysis might vary markedly. The redox state of each individual proteolytic effector is the net result of all individual influences over its relative tendencies to be oxidized or reduced by the many cell factors involved. A particular set of redox-active cell factors could influence each redox-sensitive target entity differentially (Claiborne *et al.*, 1999). It is conceivable that an increased flow of reductive energy through the network by unknown genetic, regulatory, or metabolic mechanisms could cause an increase beyond the normal reductive status of some individual components or generalized "reductive stress."

Functional demand and muscle redox status

Various studies suggest that extreme increased functional demand on muscle is associated with increased demand on the cell redox network to meet various metabolic needs (Verbundt and Van Der Laarse, 1997). One possibility is that increased GSH oxidation is required to metabolize increased ROS as by-products of increased O_2 utilization; however, other requirements for increased reductive energy under increased functional demand are also conceivable. Some studies have measured total blood GSH and GSH disulfide levels, and their ratios before and after extreme exercise in humans and rats. When oxidized intracellular GSSG accumulates beyond the ability of tissues to reduce it back to GSH, some tissues export GSSG into the extracellular fluid and blood. Therefore, increased blood GSSG following exercise reflects a pro-oxidative condition in various muscle tissues (reviewed in Vina *et al.*, 1995; Sen, 1998, 1999; Atalay and Sen, 1999). In humans, oxidized blood GSSG levels are higher after treadmill exercise. Levels of increased GSSG were linearly correlated with increased blood lactate levels. After rats were exercised on a treadmill, blood GSH decreased from 1,433 to 1,175 μM, and GSSG increased from 23 to 105 μM. The blood GSSG/GSH ratio increased from 17 to 90×10^{-3}. In these experiments, the intracellular redox status of cardiac and skeletal muscles are unknown; however, an appreciable change is suggested by such *in vivo* results on extracellular GSH status (Vina *et al.*, 1995). In the loaded perfused heart, the NADPH/$NADP^+$ ratio is unchanged by moderate functional stimulation. However, more extreme functional stimulation does induce a decrease in the NADPH/NADP redox ratio (Heineman and Balaban, 1993; Scholz *et al.*, 1995). The GSH status of the heart can also change in relation to moderate functional demand (Chen *et al.*, 1995; Verbundt and Van Der Laarse, 1997; Atalay and Sen, 1999).

Selective control of energy flow through network components

Thus far, there is little information on possible "switches" over the flow or reductive energy to proteolysis independent of overall supply–demand balance. Various thioredoxin, glutaredoxin, and protein disulfide isomerase chain systems and lipoic acid can be compartmentalized in mitochondria, ER, choloroplast, periplasm, *etc.* (Debarbieux and Beckwith, 1998, 1999; Bader *et al.*, 1999; Hirasawa *et al.*, 1999; Pedrajas *et al.*, 1999). The various thioredoxins, glutaredoxins, and protein oxidoreductases mediating pathways of Fig. 2 do not appear to exhibit much specificity for the substrates they reduce. Selective regulation of reductive energy flow through individual branches to protein reduction has not yet been demonstrated other than compartmentation. Selective nitrosyltions might be a candidate for selective sulfhydryl protease control (Rossig *et al.*, 1999; Sehajpal *et al.*, 1999). Alternative pathways demanding NADPH include NADPH oxidase, the mixed function oxygenase-reductase system, and others that are omitted here because little information exists as to how these competing pathways might interact with the overall network or protein redox (Babior, 1999).

General mechanisms of experimental pro-oxidative agents

Experimental or pharmacologic interference with the entire cell redox network or branches can result from: (1) consumption of reducing energy at a rate greater than the ability of the chains to transfer it from glucose, *i.e.*, supply-demand imbalance; (2) diversion of reducing energy from normal pathways to artificial elec-

tron sinks by redox cycling agents, *e.g.*, "short-circuiting" to oxygen; (3) inhibition of various transferring enzymes in the network independent of the overall reductive status of the system (Starke *et al.*, 1997; Stubauer *et al.*, 1999); and (4) some combination. Some agents such as alloxan are believed to be both electron sinks, as well as redox cycling agents.

The features of some experimental agents or drugs are particularly useful among the many redox-active agents known to intervene in mammalian tissues, including perfused myocardium. Diamide is known to promote the formation of many protein-mixed disulfides in the rat heart (Grimm *et al.*, 1985). DTT can reverse most of these diamide-induced disulfides, similar to endogenous reductases. Among endogenous metabolites DHA is a strong sulfhydryl oxidant, a protein ligand, a feasible experimental tool, and also a potentially important intracellular and intercellular redox signal (see below). Although many redox cycling agents exist, few are suspected of transferring energy exclusively from a specific redox chain component to an electron sink such as oxygen. Primaquine action might include a redox-cycling mechanism, as well as other mechanisms (Vasquez-Vivar and Augusto, 1992). Primaquine provides valuable comparison of *in vitro* effects with much background in human pharmacology. These diverse agents are nontoxic at exposure levels that are effective in intervening in mammalian redox metabolism (Lockwood, 2000). Accordingly, diamide-DTT or DHA-DTT are among the most useful redox probes for proteolysis in isolated mammalian tissues; primaquine is also a valuable *in vivo* tool.

Although many drugs are redox-active (Ramakrishna *et al.*, 1990), few or none have proven to be completely selective for particular enzymes of the redox system (Arner *et al.*, 1995; Nordberg *et al.*, 1998). Due to the interrelationships of redox chains, initial pharmacologic interventions can cause secondary perturbations in the entire network (Sen, 1998).

Selective metabolic intervention in high-energy phosphate and redox networks and protein degradation

Glucose as sole experimental substrate feeds both the glycolytic pathway and the pentose phosphate pathway, thereby maintaining the cell content of reductive energy via redox chains and ATP via glycolysis and below (Fig. 3) (Lockwood, 1999). Accordingly, an experimental supply–demand imbalance in cell ATP or redox status can be caused by decreasing their production and/or increasing demand for their function; however, a sufficient time period must be provided to establish a steady state between input and output. With glucose as the sole experimental substrate, moderate glucose insufficiency can be met by compensatory increases of both hexokinase or G-6-PD activities so as to maintain their respective products. However, when the intracellar glucose is decreased to an extremely low level, the rate-limiting enzymes are unable to compensate further. The redox chains and high-energy phosphate system have buffering capacity to overcome temporary supply–demand imbalance; however, the kinetics of their respective depletions differ markedly. Muscle cell ATP stores can be briefly supplemented by stores of phosphocreatine; however, upon acute ATP deficiency, functional decline begins within minutes. In contrast, the major redox reservoir of several millimolar GSH can buffer a supply–demand imbalance for prolonged periods following a decrease in reductive supply under normal metabolic demand for reductive energy. Accordingly, with glucose as the sole metabolic substrate, acute total glucoprivation can result in cell death from ATP deficiency under basal demand within minutes, whereas cell reducing chains are not simultaneously changed. A sufficient excess of exogenous pro-oxidant can deplete GSH and other redox chain components within several minutes; however, this does not reflect normal *in vivo* metabolic demand for redox chain function. The cell redox stores are only slowly depleted upon severe total nutrient deficiency in the absence of exogenous pro-oxidant to increase basal demand for their function.

Selective intervention in the cell redox network versus high-energy phosphate network can be achieved with diamide as pro-oxidant and sodium azide as uncoupler of mitochondrial oxidative phosphorylation, respectively (Lockwood, 1999); however, several reservations are implied. Stage 2 of the pentose phosphate pathway can return the ribose phosphate from Stage

1 via interconversion pathways. However, during severe inhibition of ATP production, the rate of metabolic return from the pentose phosphate pathway is grossly insufficient to sustain ATP levels. Conversely, upon sufficient pro-oxidative redox intervention, the cell is not able to provide sufficient reducing energy from the normal sources or any compensatory interconversion sources so as to maintain cell redox ratios.

Interpretation of experimentation with ATP-dependent and redox-dependent metabolic parameters under selective depletion-repletion

The fundamental properties of the high-energy phosphate and redox networks lead to several expectations in experimentation with a cell process that is hypothetically dependent upon only one network, such as protein degradation (Fig. 3, and summarized below). Using glucose as sole substrate, a promptly responding ATP-dependent process should start to decline shortly after acute nonischemic glucoprivation, as phosphocreatine, glycogen stores, and ATP are depleted within minutes under basal demand. In contrast, under identical conditions, a hypothetical redox-dependent process might not start to decline for some time after acute glucoprivation because basal metabolic reductive demand does not deplete redox stores and chains for periods longer than several hours. The experimental problem of delayed depletion of redox chains can be approached by studying the glucose dependence of the kinetics of reversal of a hypothetical redox-dependent parameter following washout of a suitable pro-oxidant agent (see Lockwood, 1999). In the continued presence of adequate glucose, a promptly ATP-dependent process should decline within minutes when a mitochondrial uncoupler inhibits oxidative phosphorylation and depletes ATP. However, in theory, a redox-dependent process need not decline under inhibition of oxidative phosphorylation and depletion of ATP until metabolic chaos precedes cell death.

REDOX-RESPONSIVE PROTEOLYTIC EFFECTOR MECHANISMS

Redox-responsive proteases

Protease redox-responsiveness can be mediated either via the active site or remote sites (Davis *et al.*, 1999; Rossig *et al.*, 1999). In theory, redox mechanisms might involve direct interactions of the cell redox system with the protease or indirect interactions mediated by control of metal ions, metabolites, or substrate properties. Members of sulfhydryl, serine, and carboxyl protease classes have been shown to be directly or indirectly responsive to their redox environment, including metal and NO interactions. Demonstrated redox-responsive sulfhydryl protease reactions include protease-mixed disulfide formation, nitrosylations, metal ion interactions, and reactions or associations with endogenous redox-active metabolites. Despite the existence of reductive peptide cleavage mechanisms, it has not been shown that the reduction of proteases transfers a stoichiometric reducing equivalent into a peptidolytic reaction mechanism. It rather appears that a reduced protease continues to function until some oxidative event or metal binding changes the active site by mechanism(s) requiring reductive energy or metal removal to reverse. It is conceivable that the oxidative inactivation of some redox-sensitive proteolysis is mediated by specific or group-specific inactivating systems that are opposed by reductive processes, *e.g.*, DHA, menaquinone, ubiquinone, flavins, NO, metal binding, etc. (Loechler and Hollocher, 1975; Baum *et al.*, 1996; Banhegyi *et al.*, 1998; Bader *et al.*, 1999; Debarbieux and Beckwith, 1999).

Redox-responsive cysteine proteases

Papain illustrates two distinct mechanisms of sulfhydryl protease reactivation following reversible formation of active site mixed disulfides: disulfide-sulfhydryl exchange and thioredoxin-mediated reduction (Table 1). A papain-mixed disulfide can exchange its oxidized redox state with a surrounding nonprotein monosulfhydryl to yield a reduced papain monosulfhydryl and a nonprotein disulfide, provided that the nonprotein disulfide is more stable than the protease-mixed disulfide (Table 1). An artificial papain-mixed disulfide can be formed by reaction with methyl methane thiosulfonate: papain-S-S-CH_3 (Singh *et al.*, 1995, and see other sections in this review). This inactive protease-mixed disulfide readily yields its oxidized status to a large variety of sur-

rounding monosulfhydryls, thereby resulting in a reduced activated papain sulfhydryl and a more favored nonprotein mixed disulfide. Monosulfhydryls reported to exchange redox status with papain S-S-CH_3 include cysteine, cysteamine, glutathione, penicillamine, 2-mercaptoethanol, and 3-mercaptopropanoic acid, which span a wide range of pK_a values. Interestingly, the papain disulfide can also be reduced by reduced proteins, revealing exchange of redox status directly between an oxidized inactive protease and a reduced potential substrate. Whether reduced substrate proteins can reductively activate proteases *in vivo* is unknown. Second, the reduction of papain-mixed disulfide can also be mediated by the purified reconstituted NADPH dependent-thioredoxin reductase/thioredoxin system without the necessity of a surrounding monosulfhydryl (Stephen *et al.*, 1993; Kerblat *et al.*, 1999) (Fig. 2). In this case, the reduction of the protease active-site disulfide is coupled to the intramolecular oxidation of thioredoxin disulfhydryls, comparable to the DTT reduction.

Other than protease-mixed disulfide formation, a sound theoretical basis exists for speculation as to intramolecular redox interactions among the active site amino acids of some sulfhydryl proteases. The cysteine thiolate anion of many proteases is believed to interact with a vicinal imidazolium cation of histidine (Storer and Menard, 1994). This cysteine–histidine interaction can exist in an active or inactive intramolecular state. Each of these two vicinal amino acid side groups is also highly interactive with metals alone; the close vicinal positions of these amino acid side groups should greatly enhance their shared metal interactions. In addition, the amino acids of this catalytic pair can oxidize. The extreme metal sensitivity of proteases such as cathepsin B is most likely explained by successful interaction of the metal ion with the critical cysteine–histidine pair despite the presumed barrier to metal entry and cytoplasmic metal buffers. It is obvious that metal ions can be introduced by substrates, metabolites, or inhibitors that simultaneously bind the recognition site of the protease and also a metal ion. It is not presently known whether the valence state of a metal ion might influence this metal–protease interaction or whether the cell redox system might influence the active site status via the redox state or coordination state of some metal ions. Cations of zinc, copper, and iron are all interactive with cysteine and histidine. An example of endogenous metal ion inhibition of protease activity is provided by partial iron inhibition of the malarial parasite protease falcipain because hemoglobin is degraded in oxidatively stressed erythrocytes.

The activity of the ubiquitous sulfhydryl protease calpain is inhibited by pro-oxidative agents; however, its autocatalytic activation is not simultaneously inhibited (Guttman *et al.*, 1997). Because calpain contains a calmodulin structure, this enzyme might provide an example of multiple influences over the activity of a single protease, *i.e.*, both individual controls and coordinated redox controls shared with other proteases. Nonetheless, there is no current evidence that intracellular calpain activity is actually controlled by redox chains.

Lipoic acid can potentiate the activation of caspase activity in association with apoptosis (Sen *et al.*, 1999); and NO can inhibit (Rossig *et al.*, 1999).

Dozens of viral proteases are sulfhydryl enzymes that are inhibitable by sulfhydryl reagents (Gorbolenya and Snijder, 1996; Barett *et al.*, 1998). In addition to processing of virally encoded proteins, a large variety of other protease functions have been suggested in the strategies of various viral life cycles (Gorbolenya and Snijder, 1996; Barret *et al.*, 1998). Suggested viral protease functions range from limited proteolysis of specific viral or host proteins to general degradation of many host proteins. Some viral genomes code glutaredoxin; and it seems likely that some viral proteases might be responsive to host redox metabolism (Ahn and Moss, 1992; Davis *et al.*, 1996, 1997, 1999; Gorbolenya and Snijder 1996; Gvakhara *et al.*, 1996).

Although definitive evidence is scarce, it appears that various sulfhydryl proteases can differ markedly in their tendencies to inactivate oxidatively. Some sulfhydryl proteases can function under extracellular conditions, whereas others promptly exhibit little or no activity without an effective reductant. All sulfhydryl proteases can be inactivated by harsh sulfhydryl oxidants; however, some proteases such as cathepsin B appear to lose activity spon-

taneously and readily under a variety of conditions removed from the intracellular reducing environment. A differential effectiveness of the cell reductive network in reactivating various proteases is another speculative mechanism of differential redox-responsiveness. Diversity of the relative intrinsic rates of protease inactivation versus reactivation might provide the theoretical basis for selective controls by individual net redox sensitivity. A particular set of intracellular redox circumstances could exert different effects on caspases, calpains, lysosomal cathepins, or other redox-responsive proteases involved in various cell functions.

In summary, the kinetics of redox-responsiveness of various proteases might be multifactorial and very complex, involving specific active site interactions, nonspecific active site sulfur oxidations, nitrosations, metal ion binding, and various reactions at remote sites. For purposes of future experimentation, it seems advisable to define the concept of net redox sensitivity of a particular protease as the balance between opposing oxidative versus reductive influences. It is obviously difficult to determine relative rates of intracellular protease inactivation versus reactivation with the use of a purified enzyme assay system.

Endogenous activators of sulfhydryl proteases

Sulfhydryl protease activity can be modulated by both nonspecific active site influences or specific individual controls such as activation of calpains or caspases. Several candidates have been considered endogenous activators of sulfhydryl protease active sites.

Other than its protective antioxidant action, GSH has not generally been considered an endogenous protease activator. The activating action of GSH is 100-fold less potent than DTT and even supraphysiologic 10 m*M* GSH does not produce the maximal activation observed with DTT. However, in our routine assays, the submaximal stimulatory action of GSH on cathepsin B is steeply concentration dependent over the physiologic range of 1–6 m*M*. Physiologic GSH can provide approximately 25% of the activating action observed with 5 m*M* DTT (Fig. 4). Whereas DHA is inhibitory, GSH can certainly provide an appreciable amount of nonenzymatic activation of cathepsin B, and presumably some other proteases. It is likely that glutaredoxin can enhance this nonenzymatic action of GSH; however, glutaredoxin has not yet been characterized as a protease activator.

DHLA is synthesized from mitochondrial octanoic acid and subsequently is conjugated to lysines of oxidoreductases as a prosthetic group. Mitochondria undergo extensive fusions with the lysosomal and Golgi–ER membrane systems; therefore, various forms of lipoic acid must be found throughout the cell membrane systems. Using a reconstituted enzyme system, it has been found that DHLA can reduce thioredoxin (Holmgren, 1979b). DHLA might provide an alternative pathway of thioredoxin and protein reduction (Arner *et al.*, 1996). Additional roles of unincorporated DHLA as a redox shuttle have been suspected. Biologically relevant amounts of DHLA correspond approximately to 1–10 μM. DHLA has been investigated as a candidate activator of caspases and apoptosis (Sen *et al.*, 1999).

In our experience, a low 1.5 μM DHLA concentration can activate standard cathepsin B assay using fluorogenic peptide derivatives (Fig. 5). Thus, DHLA is at least as potent as reported 5–10 μM physiologic thioredoxin concentrations, and more potent than DTT in activating cathepsin B. Infusion of 100 μM extracellular DTT into the perfused heart causes essentially no effect on basal proteolysis; however, 100 μM DHLA causes a very large prompt increase over basal proteolysis followed by the death of the organ within 60 min. These supraphysiologic pharmacologic conditions suggest a direct activation by DHLA. *In vivo* protease activation by DHLA could be direct or involve a thioredoxin intermediate.

Thus, the identical model protease cathepsin B can be experimentally activated by GSH, DHLA, and thioredoxin. Glutaredoxin will most likely be shown to enhance the spontaneous action of GSH enzymatically. Such results suggest alternative pathways of protease activation, perhaps in association with compartmentation of reducing systems. The physiologic concentration dependencies of protease activation by endogenous reductants suggests

that these systems might prove to be actual controls as well as protective antioxidants. DHLA appears to be the most potent activator thus far.

Redox-responsive aspartate protease

An advance of great theoretical importance is the finding that HIV-1 aspartate protease is redox-responsive via sulfhydryl groups remote from the active site (Davis *et al.*, 1996, 1997, 1999; Mahmood *et al.*, 1998; Basu *et al.*, 1999); and NO might be involved (Sehajpal *et al.*, 1999). Each subunit of this homodimeric protease has a highly conserved cysteine that can inhibit the assayed activity in response to diamide, glutathiolation or metal ions. Glutaredoxin is detected within HIV-1 and is suggested to regulate the protease activity (Davis *et al.*, 1996, 1997, 1999). The exact role of the conserved sulfhydryls and nitrosothiols in the viral life cycle and relation to host reductive status is unknown. Intracellar mechanisms possibly inactivating and reactivating nonactive site protease sulfhydryls might also include the same mechanisms described for active site sulfhydryls (Table 1). Importantly, the HIV-1 protease was characterized prior to knowledge of its redox-responsiveness, therefore, some other known proteases might also be found to be redox-responsive via sites remote from the catalytic mechanism.

Possible redox-responsiveness of serine proteases

Serine proteases have been found indirectly redox-responsive via their responsiveness to endogenous metals. A new serine protease inhibitor acts by recruiting low amounts of endogenous Zn^{2+} (Katz and Luong, 1999). It appears that this agent acts by defeating the unknown intracellular controls permitting metal-sensitive serine proteases to function in the presence of excess of inhibitory metals. It is possible that the inhibitor–metal complex binds the enzyme active site with greater probability than the metal alone or, perhaps prevents removal of the metal by cell reductive or metal binding systems. The cytomegalovirus protease can be inactivated by a flavin mechanism, although such actual *in vivo* control is uncertain (Baum *et al.*, 1996).

Redox-unresponsive proteases

Proteases mediating redox-unresponsive proteolysis are not yet identified; however, metalloendoproteases are among likely candidates. Whereas Zn^{2+} strongly inhibits sulfhydryl proteases, Zn^{2+} is required for the activity of various metalloendoproteases, carboxypeptidases, and aminopeptidases found in all cell types. Some types of serine or carboxyl proteases might also mediate redox-unresponsive proteolysis.

Redox-dependent protease inhibitors

Dormant plant seeds store monosaccharides and amino acids in the form of polysaccharides and proteins. Upon seed germination, these nutritional storage macromolecules are broken down as a temporary sources of monomers in seedlings. Breakdown of both types of storage macromolecules in plants is partly under control of protease and amylase inhibitors that are activated during seed formation and inactivated upon germination (Wong *et al.*, 1995). These peptide inhibitors of macromolecular breakdown are only active when oxidized and are inactivated when their disulfide bonds are reduced. It has been suggested that the onset of reductive energy flow during germination controls storage protein and polysaccharide breakdown via redox control of their respective inhibitors. The purified reconstituted NADPH-dependent thioredoxin system can reduce these plant protease and amylase inhibitors (Wong *et al.*, 1995). Kunitz-type mammalian protease inhibitors can also be slowly inactivated by reduction of their internal disulfide bonds; however, such a controlling role in mammalian proteolysis is unknown. In yeast, thioredoxin can be found in a heterodimer with a protease inhibitor, although the significance is unknown (Xu and Wickner, 1996; Slusarewicz *et al.*, 1997). A search for DHA reductase activity in chloroplasts revealed a protein that was subsequently found to contain a Kunitz-type protease inhibitory sequence (Morell *et al.*, 1997).

Substrate protein reduction and unfolding

Unfolding of a globular protein or failure to fold can increase the exposure of peptide bonds

to effective collisions and reactions with proteases by 10- to 100-fold (*e.g.*, Stathakis *et al.*, 1997). It is suggested that only unfolded substrate can be translocated through the proteasome (Baumeister *et al.*, 1998); however, essentially any protease has a markedly increased probability of nonspecific reaction with an extended peptide remaining in solution, including immunoglobulins (Kerblat *et al.*, 1999). There is no evidence that all or most folded proteins must be reduced prior to proteolytic cleavage by most proteases. Reduction of protein disulfides can be mediated by thioredoxins, glutaredoxins, or protein disulfide oxidoreductases. The effects of disulfide reduction and metal ion removal have been studied with insulin degradation (Arquilla *et al.*, 1978; Holmgren, 1979a,b; Jung and Thomas, 1996). Precipitated proteins, such as bacterial inclusion bodies, are less likely to be degraded than soluble proteins, regardless of redox state.

Advances in the redox reactions and compartmentation of protein folding have been recently reviewed (Rietsch *et al.*, 1997; Debarbieux and Beckwith, 1998, 1999; Bader *et al.*, 1999; Bessette *et al.*, 1999). It is not known whether the systems reducing structural disulfides might also be involved in control of protease active sites. However, substrate protein oxidation might be a factor in redox responsiveness of the Golgi-ER degradation observed from 0 to 3 hr following biosynthetic labeling of mammalian cells.

Removal of inhibitory metal ions from substrate proteins and proteases

The surface of many proteins contains metal ions bound to specific, high-affinity sites or by weaker electrostatic interactions. Zn^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+} are all strongly inhibitory to some sulfhydryl and other proteases at physiologic levels. The many Zn^{2+} ions bound to insulin are suspected of an influence on its degradation by some proteases, although few other proteins have been similarly studied (Arquilla *et al.*, 1978). Disulfhydryls can form high-affinity complexes with various endogenous and nonbiologic metals (Cornell and Crivaro, 1972; Sigel *et al.*, 1978; Reyes, 1996). Monosulfhydryls can buffer metals. Because substrate-bound metals can deliver an inhibitory agent to an enzyme active site, the known stimulatory action of disulfhydryl agents on proteases might include interactions with metals on substrate as well as enzyme (Stubauer *et al.*, 1999).

Redox-dependent protein conjugation reactions

Several distinct enzymatic systems serve to conjugate several small protein tags to many other proteins (Mizushima *et al.*, 1998). Among the multiple functions of such protein conjugation systems are proposed influences on some steps in the overall degradation of proteins. Suggested functions of the protein conjugation systems include influences on membrane movement, formation of autophagic vacuoles, and subcellular protein localization, all of which might influence protein turnover. Another suggested function of protein conjugation systems involves the interaction of conjugated substrates with proteases, although no clear set of rules has been elucidated thus far. In addition to ATP, the protein conjugation reaction sequence requires reduced enzyme sulfhydryls forming intermediate thioester bonds during the overall reaction. The protein conjugation systems are inactivated by thiol-reactive agents. Activities of the ubiquitin activation enzyme and ubiquitin-conjugating enzyme have been suggested to be correlated with GSH redox status (Jahngen-Hodge *et al.*, 1997). Glutathiolation-dethiolation reactions were proposed in regulating the function of the ubiquitin conjugation system (Shang *et al.*, 1997; Obin *et al.*, 1998).

Redox requirement of membrane–cytoskeletal function

As morphologically observed, diamide and other pro-oxidants can cause profound structural changes in the cell vacuolar–cytoskeletal system (Leung and Chou, 1989). Thioredoxin is believed to influence overall vacuolar–cytoskeletal function by unknown mechanisms (Xu and Wickner, 1996; Slusarewicz *et al.*, 1997). Thus, in addition to above reactions, some of the reductive requirement for proteolysis could somehow be associated with cell cytokinesis, membrane–vacuolar movements, and vacuolar substrate acquisition. Some of the above-

reviewed protein conjugation reactions might also be involved in membrane traffic (Mizushima *et al.*, 1998). Various other studies of specific individual proteins have suggested an effect of redox status on Golgi–ER protein translocations and/or degradation (Torbrella and Ploegh, 1998; Young *et al.*, 1993).

Autocatalysis and nonenzymatic cleavage of peptide bonds

Some proteins can undergo internal autocatalytic cleavage of peptide bonds by several mechanisms (Guttman *et al.*, 1997; Perler *et al.*, 1997). However, the extent of internal autocatalysis as an initiating event in general cell protein degradation is unknown. It has not been determined whether some types of internal autocatalysis might be influenced by the redox environment. Various agents can nonenzymatically cleave peptide bonds by input of reductive energy, although reductive cleavage is not known to be operative as a protease mechanism. For example, sodium borohydride can cause reduction of protein disulfide bonds and also cleavage of peptide bonds (Yakulis *et al.*, 1968). Cyanogen bromide causes reductive cleavage near methionine, although such harsh conditions are not relevant to *in vivo* proteolysis. Ascorbic acid with metals and oxygen can cause slow nonenzymatic peptide cleavage, perhaps by unknown secondary reaction mechanisms. Metals can form a true complex with the peptide bond; therefore, in some reaction mechanisms, metals might serve as a conduit for transfer of energy into or out of the bond. The extent of intracellular peptide cleavage independent of proteases is unknown, but perhaps a small amount of either redox-dependent or redox-independent proteolysis might be mediated nonenzymatically.

REDOX-RESPONSIVE AND REDOX-UNRESPONSIVE SUBCOMPONENTS OF MYOCARDIAL PROTEIN DEGRADATION AND ATP NONRESPONSIVENESS OF BOTH

The perfused heart offers unique advantages in some metabolic studies not possible in cultured cells or in *in vivo* tissues. Recent improvements in technique permit vigorous contractile function over more than 7 hr (Lockwood, 1985, 1999). Under aortic perfusion with an external peristaltic pump at constant flow rate, the circulation of a perfused heart is maintained constant independent of the presence or absence of its own contractile function. Total protein degradation in the present nonloaded preparation is independent of electrically paced heart rate up to the maximally attainable rhythmic rate approaching 400 beats per min, *i.e.*, essentially a rhythmic flutter. Conversely, protein degradation is also unchanged when the heart is stopped under depolarized conduction block with 25 m*M* perfusate K^+, *i.e.*, cardioplegia. Therefore, total protein degradation in the nonloaded heart is not linked to short-term changes in contraction–relaxation, although the loaded preparation presumably differs due to metabolic sequellae of functional demand.

Hearts are biosynthetically labeled *ex vivo*; however, results are indistinguishable using injection of [^{3}H]leucine into the rat several hours prior to perfusion. Cytoplasmic amino acid pools are exchanged with a half-time of 0.5 min, over a 20-min preliminary washout period. Reincorporation of radiolabeled postcursor is prevented with 1 m*M* nonradioactive leucine or cycloheximide with identical results, except for the eventual toxicity of cycloheximide after 2 hr. Under nonrecirculating perfusion the total release of [^{3}H]leucine is measured in the effluent at 1- or 2-min intervals with a fraction collector. Simultaneous ventricular function and protein degradation are compared using a fluid-filled ventricular catheter connected through a pressure transducer so as to measure the ventricular pressure pulse. Concentrated experimental agents are infused by pump into the flow of nonrecirculating perfusate. Ventricular dysfunction occurs when biopsied ATP declines to approximately 40% of basal *in vivo* levels under infusion of sodium azide. Effective diamide or DHA concentrations cause essentially no apparent contractile dysfunction or toxicity over 1 hr. Additional features have been previously described (Lockwood, 1997, 1999; Thorne and Lockwood, 1993).

Evidence for redox responsiveness and ATP nonresponsiveness of major intracellular proteolytic pathways

Against the above background, evidence for the maintenance of major subcomponents of total myocardial protein degradation by reducing energy can be partially summarized (further discussed in Lockwood 1997, 1999; Thorne and Lockwood, 1993): (1) Low nontoxic diamide concentrations cause more than 75% inhibition of total myocardial proteolysis beginning with minutes. Diamide action was inhibitory at all effective concentrations with no biphasic responses. Most of such diamide inhibition is metabolically reversible within 1–2 hr of discontinuation (Fig. 3). Therapeutic concentrations of primaquine noninjuriously inhibit most, but not all, of the myocardial proteolysis that is inhibited by diamide. (2) DHA at physiologic exposure levels of 5 μM causes no inhibition of proteolysis; however, supraphysiologic levels slightly above 15 μM mimic the antiproteolytic action of diamide (Fig. 4). (3) The antiproteolytic actions of diamide or DHA can be largely reversed under continued oxidant exposure by simultaneous infusion of DTT, which is known to reduce both oxidative agents and potential cell targets of their oxidative actions (Fig. 4). (4) Inhibition of proteolysis under diamide is not accompanied by change in measured ATP content of ventricular biopsies or ventricular contractile dysfunction. (5) Prolonged exposure to the mitochondrial uncoupler sodium azide can cause nearly lethal 60% ATP depletion and contractile dysfunction without changing total protein degradation. (6) Acute glucoprivation or glucose analogues can cause contractile dysfunction and presumptive ATP depletion without immediately changing protein degradation over more than 1 hr; however, acute glucoprivation greatly accelerates the onset of antiproteolytic action of either diamide or DHA. (7) Reversal of diamide antiproteolytic action is strongly dependent upon the myocardial glucose supply. (8) Under the above experimentation, a major subcomponent of proteolysis is simultaneously unchanged by redox agents that permeate the entire cell. (9) Much of the redox-responsive proteolytic subcomponent can also be inhibited by the redox-inactive sulfhydryl protease inhibitor E-64; however, the redox-unresponsive subcomponent is simultaneously uninhibited. (10) The reversible intracellular antagonism between DHA and DTT corresponds to complete inactivation of purified sulfhydryl protease activity (cathepsin B) by DHA followed by reactivation by DTT in enzyme assays.

Possible opposing roles of DHA and GSH in regulation of proteases and proteolysis

In addition to an experimental tool, it is conceivable that DHA is actually a redox messenger. Much of the cell import and export of AA/DHA and GSH/GSSG and details of interorgan metabolism remain to be described; however, it is obvious that these two components of intracellar redox chains somehow serve in metabolic communication between mammalian cells (Sies, 1977; Griffith and Meister, 1979; Vera *et al.*, 1993; Welch *et al.*, 1995; Banhegyi *et al.*, 1997, 1998). It is almost certain that the AA/DHA system of mammals serves undiscovered function(s). Interestingly, the same cell reductases that reduce DHA to AA also reduce GSSG to GSH and oxidized proteins to reduced proteins (Basu *et al.*, 1979; Wells *et al.*, 1990; Washko *et al.*, 1992; May *et al.*, 1997, 1998; Washburn and Wells 1999a,b) (Fig. 4). DHA is a highly reactive oxidant that can reversibly inactivate reduced sulfhydryl proteases or oxidize GSH to GSSG (Rose and Bode, 1992; Winkler, 1992; Krauth-Sigel and Ludeman 1996; Lockwood, 1999; Vethanayagam *et al.*, 1999). Ascorbic acid can spontaneously lose an electron to become the radical semi-dehydroascorbic acid (SDA) (Fig. 4). The SDA radical quenches radical chain reactions. SDA can spontaneously lose a second electron to become the ring triketone DHA. Some ketones are excellent general protease inhibitors. DHA can either undergo ring cleavage or be reduced enzymatically or nonenzymatically back to AA. Thus, the AA/SDA/DHA system exists in a state of cyclic exergonic oxidation and active endergonic reduction at the expense of cell reducing energy. Nonenzymatic reduction of DHA to AA can be coupled to oxida-

tion of GSH to GSSG (Winkler, 1992; Vethanayagam *et al.*, 1999) (Fig. 4). Whereas DHA might oxidatively inactivate proteolysis, AA might serve as an antioxidant protecting proteolytic machinery. Intracell AA is not a trace substance, but is rather 1 m*M*. The intracellular DHA concentration is uncertain, but probably is in the low micromolar range under normal reduced cytoplasmic conditions. The cell content of AA can be highly variable; and intracellular DHA levels might increase under grossly pro-oxidative conditions. The extracellular DHA concentration is approximately 5 μM. A passive carrier takes up extracellular DHA; and intracellular reductases rapidly reduce it to AA, thereby creating the transmembrane concentration ratio of 1 m*M* intracellar AA to several micromolar extracellar DHA (Vera *et al.*, 1993). The DHA transport carriers have been identified as facilitative glucose carriers.

An involvement of the redox chains of the ER in protein redox has been suggested, including DHA, ubiquinone, and menaquinone as electron acceptors in protein oxidations (Banhegyi *et al.*, 1997; Bader *et al.*, 1999; Debarbieux and Beckwith, 1999). In the myocardium, rapid turnover proteolysis or Golgi–ER proteolysis can be strongly inhibited by menaquinone (menadione) at micromolar concentrations. However, this action is not specific for menaquinone insofar as benzoquinone or DHA also inhibit rapid turnover proteolysis (Lockwood, 1997, 2000). Thus, various endogenous oxidants can certainly decrease proteolysis; however, it is not known whether this effect is exerted primarily on substrate properties, proteases, or some other effector mechanism. The immediate antiproteolytic action of 20 μM DHA suggests direct protease inhibition prior to appreciable GSH depletion.

Degradative contribution to net change in tissue protein content

The adjustment of muscle protein mass to functional demand is a fundamental property of muscle. Demand-driven myocardial hypertrophy or disuse atrophy of skeletal muscle can be associated with pathogenic states not reviewed here. The extent of degradative contribution to net changes in muscle mass is considerable, although quantitatively uncertain. A net increase of 40% in protein mass corresponds to pathogenic hypertensive or obstructive myocardial hypertrophy. If the observed redox-responsive degradative fluctuation of 75% were sustained under constant synthesis, the eventual result would be a proportional 75% steady-state gain in represented proteins after five half-lives of the slowest turnover proteins. The exact relationship between myofibrillar assembly-disassembly and protein synthesis-degradation is uncertain; and hypertrophy involves multiple factors in addition to intracellular protein degradation. However, in theory, redox-responsive proteolysis could be a major contributing factor in regulating muscle protein mass or subdivisions in relation to functional demand.

CONCLUSIONS

The above survey of many species and cell types reveals an unappreciated diversity of redox-responsive proteolytic effectors and controls in addition to sulfhydryl proteases. Speculatively, each redox-responsive proteolytic effector mechanism might have evolved its own particular net responsiveness to the prevailing cell redox status, *e.g.*, individual control of proteases, protease inhibitors, substrate redox, metal ion interactions, substrate conjugating systems, membrane-cytoskeletal function, *etc.* However, it appears that redox-responsiveness is the only demonstrated factor that can transcend the lysosomal, Golgi–ER, and some extravesicular proteolysis in a common coordinating mechanism under some experimental conditions. It is remarkable that large changes in cell protein degradation can be caused by pharmacologic or endocrine interventions that are virtually nontoxic over prolonged periods in humans.

The cell content and function of virtually any protein or group of proteins can potentially be influenced by the catabolic component of turnover. Oxidative or reductive abnormalities might be associated with insufficient or excess

functions of the proteolytic effector mechanisms reviewed above. The number and diversity of redox-responsive proteolytic effector mechanisms might provide many specific targets for future pharmacologic interventions. Background not reviewed here suggests a wide variety of therapeutic applications of proteolytic redox pharmacology, including viral or parasite–host relationships (McKerrow *et al.*, 1993; Atamna *et al.*, 1994; Fryauff *et al.*, 1995; Bozzi *et al.*, 1996; Ginsberg *et al.*, 1996; Barret, 1997; Francis *et al.*, 1997; Golenser *et al.*, 1998; Krauth-Sigel and Coombs, 1999; McKerrow, 1999; Sehajpal *et al.*, 1999), immunologic (Droge *et al.*, 1994; Kerblat *et al.*, 1999) or endocrine disorders (Holmgren 1979a,b; Thorne and Lockwood, 1991), injury, and apoptosis (Rossig *et al.*, 1999; Sen, 1999). Hundreds of studies have pursued various redox abnormalities in Alzheimer's disease with little apparent success (Simonian and Coyle, 1996). Enzyme assemblies incorporating DHLA as prosthetic group include α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. In searches for various other causes of Alzheimer's disease, these two activities have repeatedly been found deficient without genetic linkage to the disease (Shoffner, 1997). A simple interpretation of Alzheimer's disease as failure of a redox-dependent proteolytic pathway seems unlikely; however, a contributing role of DHLA merits further consideration.

A full understanding of the redox network is the last frontier of metabolism, and will undoubtedly provide future insights into the normal and abnormal turnover of proteins.

ABBREVIATIONS

AA, Ascorbic acid; DHA, dehydroascorbic acid; DHLA, dihydrolipoic acid; DTT, dithiothreitol; G-6-PD, glucose-6-phosphate dehydrogenase; GRX, glutaredoxin; GSH, glutathione, reduced; GSSG, glutathione, oxidized; Pr•Metabolite, protein-metabolite interaction; PrSNO, nitrosylated protein; Pr•M^n, protein–metal interaction; PrSH, reduced protein; PrS-SR, protein-mixed disulfide; RSH, monosulfhydryl, reduced (general term); RSSR, disulfide (oxidized); SDA, semidehydroascorbic acid; TRX $(SH)_2$, TRX (S-S), thioredoxin, reduced, oxidized.

REFERENCES

AHN, B., and MOSS, B. (1992). Glutaredoxin homolog encoded by Vaccinia virus is a virion-associated enzyme with thioltransferase and dehydroascorbate reductase activities. Proc. Natl. Acad. Sci. USA **89,** 7060–7064.

ANDERSON, M.E. (1998). Glutathione: an overview of biosynthesis and modulation. Chem. Biol. Interaction **111,** 1–14.

APOSHIAN, H.V. (1983). Dimercaptosuccinic acid and dimercaptopropane sulfonic acid: water soluble antidotes to heavy metal poisoning. Annu. Rev. Pharmacol. **23,** 193–215.

ARNER, E.S., BJORNSTEDT, M., and HOLMGREN, A. (1995). 1-Chloro-2,4-dinitrobenzene is an irreversible inhibitor of human thioredoxin reductase: Loss of thioredoxin disulfide reductase activity is accompanied by a large increase in NADPH oxidase activity. J. Biol. Chem. **270,** 3479–3482.

ARNER, E.S., NORDBERG, J., and HOLMGREN, A. (1996). Efficient reduction of lipoamide and lipoic acid by mammalian thioredoxin reductase. Biochem. Biophys. Research Commun. **225,** 268–274.

ARQUILLA, E.R., PACKER, S., TARMAS, W., and MIYAMOTO, S. (1978). The effect of zinc on insulin metabolism. Endocrinology **103,** 1440–1449.

ARRIGO, A.P. (1999). Gene expression and the thiol redox state. Free Radic. Biol. Med. **27,** 936–944.

ASLUND, F., and BECKWITH, J. (1999a). Bridge over troubled waters: sensing stress by disulfide bond formation. Cell **96,** 751–753.

ASLUND, E., and BECKWITH, J. (1999b). The thioredoxin superfamily: redundancy, specificity, and gray-area genomics. J. Bacteriol. **181,** 1375–1379.

ASLUND, F., BERNDT, K.D., and HOLMGREN, A. (1997). Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductase of the thioredoxin superfamily determined by direct protein-protein redox equilibria. J. Biol. Chem. **272,** 30780–30786.

ATALAY, M., and SEN, C.K. (1999). Physical exercise and antioxidant defenses in the heart. Ann. N.Y. Acad. Sci. **874,** 169–177.

ATAMNA, H., PASCARMONA, G., and GINSBURG, H. (1994). Hexose-monophosphate shunt activity in intact Plasmodium falciparum-infected erythrocytes and in free parasites. Mol. Biochem. Parasitol. **67,** 79–89.

BABIOR, B.M. (1999). NADPH oxidase: an update. Blood **93,** 1464–1476.

BADER, M., MUSE, W., BALLOU, D.P., GASSNER, C., and BARDWELL, J.C. (1999). Oxidative protein folding is driven by the electron transport system. Cell **98,** 217–227.

BANHEGYI, G., BRAUN, L., CSALA, M., PUSKAS, F., and MANDL, J. (1997). Ascorbate metabolism and its regulation in animals. Free Radic. Biol. Med. **23,** 793–803.

BANHEGYI, G., MARCOLONGO, P., PUSKAS, F., FULCERI, R., MANDL, J., and BENEDETTI, A. (1998). Dehydroascorbate and ascorbate transport in rat liver microsomal vesicles. J. Biol. Chem. **273,** 2758–2762.

BARRET, M.P. (1997). The pentose phosphate pathway and parasitic protozoa. Parasitol. Today **13,** 11–16.

BARRET, A.J., RAWLINGS, N.D., and WOESSNER, J.F. (1998). *Handbook of Proteolytic Enzymes.* (Academic Press, New York).

BASU, S., SOM, S., DEB, S., MUKHERJEE, D., and CHATTERJEE, I.B. (1979). Dehydroascorbic acid reduction in human erythrocytes. Biochem. Biophys. Res. Commun. **90,** 1335–1340.

BASU, A., SEHAJAPAL, P.K., OJISTE, J.S., and LANDER, H.M. (1999). Targeting cysteine residues of HIV-1 protease by reactive free radical species. Antiox. Redox Signal. (in press).

BAUM, E.Z., DIRG, W.D., SIEGEL, M.M., HULMES, J., BEBERNITZ, G.A., SRIDHARAN, L., TABEI, K., KRISHNAMURTHY, G., CAROFIGLIO, T., GROVES, J.J., BLOOM, J.D., DIGRANDI, M., BRADLEY, M., ELLESTAD, G., SEDDON, A.P., and GLUZMAN, Y. (1996). Flavins inhibit human cytomegalovirus UL80 protease via disulfide bond formation. Biochemistry **35,** 5847–5855.

BAUMEISTER, W., WOLZ, J., ZUHL, F., and SEEMULLER, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. Cell **92,** 367–380.

BERGSTEN, P., AMITAI, G., KEHRL, T., DARIWHAL, K.R., KLEIN, H.G., and LEVINE, M. (1990). Millimolar amounts of ascorbic acid in purified human mononuclear leukocytes: Depletion and reaccumulation. J. Biol. Chem. **256,** 2684–2687.

BESSETTE, P.H., ASLUND, F., BECKWITH, J., and GEORGIOU, G. (1999). Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. Proc. Natl. Acad. Sci. USA **96,** 12703–12708.

BIAGLOW, J.E., and NYGAARD, O.F. (1973). The use of the oxidant diamide for studying the nonmitochondrial reducing capacity of Ehrlich ascites tumor cells. Biochem. Biophys. Res. Commun. **54,** 874–881.

BOZZI, A., PARISI, M., and STROM, R. (1996). Erythrocyte glutathione determination in the diagnosis of glucose-6-phosphate dehydrogenase deficiency. Biochem. Mol. Biol. Int. **40,** 561–569.

CHANCE, B., SIES, H., and BOVERIS, A. (1979). Hydroperoxide metabolism in mammalian organs. Physiol. Rev. **59,** 527–605.

CHEN, W., GABE, S., STEENBERGEN, C., and MURPHY, E. (1995). A redox-based mechanism for cardio-protection induced by ischemic preconditioning in perfused rat heart. Circ. Res. **77,** 424–429.

CHUA, B.H., and KLEINHANS, B.J. (1985). Effect of redox potential on protein degradation in perfused rat heart. Am. J. Physiol. **248,** E726–E731.

CLAIBORNE, A., YEH, J.I., MALLETT, T.C., LUBA, J., CRANE, E.J. III, CHARRIER, V., and PARSONAGE, D. (1999). Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. Biochemistry **47,** 15, 407–416.

CLELAND, W.W. (1964). Dithiothreitol: a new protective agent for sulfhydryl groups. Biochemistry **3,** 480–485.

COASSIN, M., TOMASI, A., VANNINI, V., and URSINI, F. (1991). Enzymatic recycling of oxidized ascorbate in pig heart: one-electron vs two-electron pathway. Arch. Biochem. Biophys. **290,** 458–462.

COOMBS, G.H., and MOTTRAM, J.C. (1997). Parasite proteinases and amino acid metabolism: possibilities for chemotherapeutic exploitation. Parasitology **114** (Suppl.), S61–S80.

CORNELL, N.W., and CRIVARO, K.E. (1972). Stability constants for zinc-dithiothreitol complex. Anal Biochem. **47,** 203–208.

COTGREAVE, I.A., and GERDES, R.G. (1998). Recent trends in glutathione biochemistry—Glutathione-protein interactions: A molecular link between oxidative stress and cell proliferation? Biochem. Biophys. Res. Commun. **242,** 1–9.

DAFRE, A.L., and REISCHL, E. (1998). Oxidative stress causes intracellular reversible S-thiolation of chicken hemoglobin under diamide and xanthine oxidase treatment. Arch. Biochem. Biophys. **358,** 291–296.

DAVIS, D.A., DORSEY, K., WINGFIELD, P.T., STAHL, S.J., KAUFMAN, J., FOLES, H.M., and LEVINE, R.L. (1996). Regulation of HIV-1 protease activity through cysteine modification. Biochemistry **35,** 2482–2488.

DAVIS, D.A., NEWCOMB, F.M., STARKE, D.W., OTT, D.E., MIEYAL, J.J., and YARCHOAN, R. (1997). Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. J. Biol. Chem. **272,** 25935–25940.

DAVIS, D.A., YUSA, K., GILLIAM, L.A., NEWCOMB, F.M., MITSUYA, H., and YARCHOAN, R. (1999). Conserved cysteines of the human immunodeficiency virus type 1 protease are involved in regulation of polyprotein processing and viral maturation of immature virions. J. Virol. **73,** 1156–1164.

DEBARBIEUX, L., and BECKWITH, J. (1998). The reductive enzyme thioredoxin 1 acts as an oxidant when it is exported to the Escherichia coli periplasm. Proc. Natl. Acad. of Sci. USA **95,** 10751–10756.

DEBARBIEUX, L., and BECKWITH, J. (1999). Electron avenue: pathways of disulfide bond formation and isomerization. Cell **99,** 117–119.

DI SIMPLICIO, P., CACACE, M.G., LUSINI, L., GIANNERINI, F., GIUSTARINI, D., and ROSSI, R. (1998). Role of protein—SH groups in redox homeostasis—the erythrocyte as a model system. Arch. Biochem. Biophys. **355,** 145–152.

DROGE, W., SCHULZE-OSTHOFF, K., NIHIM, S., GALTER, D., SCHINK, H., ECK, H.P., ROTH, S., and GRUNDER, H. (1994). Functions of glutathione and glutathione disulfide in immunology and immunopathology. FASEB J. **14,** 1131–1138.

FAGAN, J.M., and GOLDBERG, A.L. (1985). The rate of protein degradation in isolated skeletal muscle does not correlate with reduction-oxidation status. Biochem. J. **227,** 689–694.

FERNANDO, R.M., NANRI, H., YOSHITAKE, S., NAGATA-KUNO, K., and MINAKAMI, S. (1992). Thiore-

doxin regenerates proteins inactivated by oxidative stress in endothelial cells. Eur. J. Biochem. **209,** 917–922.

FERRARI, D.M., and SOLING, H.D. (1999). The protein disulphide-isomerase family: unraveling a string of folds. Biochem. J. **339,** 1–10.

FOYER, C.H., and MULLINEAUX, P.M. (1998). The presence of dehydroascorbate and deydroascorbate reductase in plant tissues. FEBS Lett. **425,** 528–529.

FRANCIS, S., SULLIVAN, D.J., and GOLDBERG, D.E. (1997). Hemoglobin metabolism in the malaria parasite Plasmodium falciparum. Annu. Rev. Microbiol. **51,** 97–123.

FRYAUFF, D.J., BAIRD, J.K., BASRI, H., SUMIWINATI, I., PURNOMO, RICHIE, T.J., OHRT, C.K., MOUZIN, E., CHURCH, C.J., and RICHARDS, A.L. (1995). Randomized placebo-controlled trial of primaquine for prophylaxis of falciparum and vivax malaria. Lancet **346,** 1190–1193.

GILBERT, H.F. (1984). Redox control of enzyme activities by thiol-disulfide exchange. Methods Enzymol. **107,** 330–351.

GINSBURG, H., ATAMNA, H., SHALMIEV, G., KANAANI, J., and KRUGLIAK, M. (1996). Resistance of glucose-6-phosphate dehydrogenase deficiency to malaria: Effects of fava bean hydroxypyrimidine glucosides on Plasmodium falciparum growth in culture and on the phagocytosis of infected cells. Parasitology **113,** 7–18.

GLEASON, F.K. (1996). Glucose-6-phosphate dehydrogenase from the cyanobacterium, Anabaena sp. PCC 7120: purification and kinetics of redox modulation. Arch Biochem. Biophys. **334,** 277–283.

GOLENSER, J., MILLER, J., SPIRA, D.T., KOSOWER, N.S., VANDE WAA, J.A., and JENSEN, J.B. (1988). Inhibition of the intraerythrocytic development of Plasmodium falciparum in glucose-6-phosphate dehydrogenase deficient erythrocytes is enhanced by oxidants and by crisis form factor. Trop. Med. Parasitol. **39,** 273–276.

GORBOLENYA, A.E., and SNIJDER, E.J. (1996). Viral cysteine proteinases. Persp. Drug Discov. Design **6,** 64–80.

GRIFFITH, O.W., and MEISTER, A. (1979). Glutathione: inter-organ translocation, turnover, and metabolism. Proc. Natl. Acad. Sci. USA **76,** 5606–5610.

GRIMM, L.M., COLLISON, M.W., FISHER, R.A., and THOMAS, J.A. (1985). Protein-mixed disulfides in cardiac cells: S-thiolation of soluble proteins in response to diamide. Biochem. Biophys. Acta **844,** 50–54.

GROMER, S., ARSCOTT, L.D., WILLIAMS, C.H., SCHIRMER, R.H., and BECKER, K. (1998). Human placenta thioredoxin reductase: Isolation of the selenoenzyme, steady state kinetics and inhibition by therapeutic gold compounds. J. Biol. Chem. **273,** 20096–20101.

GRONESTEJSKI, R.M., PARDEE, A.B., and GOLDBERG, A.L. (1985). The ATP-dependence of the degradation of short and long lived proteins in growing fibroblasts. J. Biol. Chem. **260,** 3344–3349.

GUTTMANN, R.P., ELEE, J.S., BELL, P.D., ISBELL, J.C., and JOHNSON, G.V. (1997). Oxidation inhibits substrate proteolysis by calpain 1 but not autolysis. J. Biol. Chem. **272,** 2005–2012.

GVAKHARIA, B.O., KOONIN, E.K., and MATHEWS, C.K. (1996). Vaccinia virus G4L gene encodes a second glutaredoxin. Virology **226,** 408–411.

HAMPTON, M.B., and ORRENIUS, S. (1998). Redox regulation of apoptotic cell death. Biofactors **8,** 1–5.

HAYES, J.D., and McLELLAN, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a coordinately regulated defense against oxidative stress. Free Radic. Res. **31,** 273–300.

HEINEMAN, F.W., and BALABAN, R.S. (1993). Effects of afterload and heart rate on NAD(P)H redox state in the isolated rabbit heart. Am. J. Physiol. **264,** H433–H440.

HIRASAWA, M., SCHURMANN, P., JACQUOT, J.P., MANIERI, W., JACQUOT, P., KERYER, E., HARTMAN, F.C., and KNAFF, D.B. (1999). Oxidation-reduction properties of chloroplast thioredoxins, ferredoxin: thioredoxin reductase, and thioredoxin f-regulated enzymes. Biochemistry **38,** 5200–5205.

HOLMGREN, A. (1979a). Reduction of disulfides by thioredoxin: Exceptional reactivity of insulin and suggested functions of thioredoxin in mechanism of hormone action. J. Biol. Chem. **18,** 9113–9119.

HOLMGREN, A. (1979b). Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254,** 9627–9632.

HOLMGREN, A. (1995). Thioredoxin structure and mechanims: Conformational changes on oxidation of the active-site sulfhydryls to a disulfide. Structure **3,** 239–243.

HOLMGREN, A., and ASLUND, F. (1995). Glutaredoxin. Methods Enzymol. **252,** 283–292.

HOLMGREN, A., and BJORNSTEDT, M. (1995). Thioredoxin and thioredoxin reductase. Methods Enzymol. **252,** 199–208.

HUBER-WUNDERLICH, M., and GLOCKSHUBER, R. (1998). A single dipeptide sequence modulates the redox properties of a whole enzyme family. Fold. Des. **3,** 161–171.

ISHIKAWA, T., CASINI, A.F., and NISHIKIMI, M. (1998). Molecular cloning and functional expression of rat liver glutathione-dependent dehydroascorbate reductase. J. Biol. Chem. **273,** 28708–28712.

JAHNGEN-HODGE, J., OBIN, M.S., GONG, X., SHANG, F., NOWELL, T.R., JR., GONG, J., ABASI, H., BLUMBERG, J., and TAYLOR, A. (1997). Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J. Biol. Chem. **272,** 28218–28226.

JAIN, A., MARTENSSON, J., MEHTA, T., KRAUSS, A.N., AULD, P.A., and MEISTER, A. (1992). Ascorbic acid prevents oxidative stress in glutathione-deficient mice: Effects on lung type 2 cell lamellar bodies, lung surfactant, and skeletal muscle. Proc. Natl. Acad. Sci. USA **89,** 5093–5097.

JORDAN, A., ASLUND, F., PONTIS, E., REICHARD, P., and HOLMGREN, A. (1997). Characterization of Escherichia coli NrdH. A glutaredoxin-like protein with a thioredoxin-like activity profile. J. Biol. Chem. **272,** 18044–18050.

JUNG, C.H., and THOMAS, J.A. (1996). S-glutathiolated

hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. Arch. Biochem. Biophys. **335,** 61–72.

KATZ, B.A., and LUONG, C. (1999). Recruiting Zn^{2+} to mediate potent, specific inhibition of serine proteases. J. Mol. Biol. **292,** 669–684.

KERBLAT, I., DROUET, C., CHESNE, S., and MARCHE, P.N. (1999). Importance of thioredoxin in the proteolysis of an immunoglobulin G as antigen by lysosomal Cys-proteases. Immunology **97,** 62–63.

KOSOWER, N.S., and KOSOWER, E.M. (1995). Diamide: An oxidant probe for thiols. Methods Enzymol. **251,** 123–133.

KRAUTH-SIEGEL, R.L., and COOMBS, G.H. (1999). Enzymes of parasite thiol metabolism as drug targets. Parasitol. Today **15,** 404–409.

KRAUTH-SIEGEL, R.L., and LUDEMANN, H. (1996). Reduction of dehydroascorbate by trypanothione. Mol. Biochem. Parasitol. **80,** 203–208.

LANGENBACH, K.J., and SOTTILE, J. (1999). Identification of protein-disulfide isomerase activity in fibronectin. J. Biol. Chem. **274,** 7032–7038.

LEE, J., SPECTOR, D., GODON, C., LABARRE, J., and TOLEDANO, M.B. (1999a). A new antioxidant with alkyl hydroperoxide defense properties in yeast. J. Biol. Chem. **274,** 4537–4544.

LEE, S.R., KIM, J.R., KWON, K.S., YOON, H.W., LEVINE, R.L., GINSBURG, A., and RHEE, S.G. (1999b). Molecular cloning and characterization of a mitochondrial selenocysteine-containing thioredoxin reductase from rat liver. J. Biol. Chem. **274,** 4722–4734.

LENZEN, S., and MUNDAY, R. (1991). Thiol-group reactivity, hydrophilicity and stability of alloxan, its reduction products and its N-methyl derivatives and a comparison with ninhydrin. Biochem. Pharmacol. **42,** 1385–1391.

LEUNG, M.F., and CHOU, I.N. (1989). Relationship between 1-chloro-2,4-dinitrobenzene-induced cytoskeletal perturbations and cellular glutathione. Cell Biol. Toxicol. **5,** 51–66.

LILLIG, C.H., PRIOR, A., SCHWENN, J.D., ASLUND, F., RITZ, D., VLAMIS-GARDIKAS, A., and HOLMGREN, A. (1999). New thioredoxins and glutaredoxins as electron donors of 3′-phosphoadenylylsulfate reductase. J. Biol. Chem. **274,** 7695–7698.

LOCKWOOD, T.D. (1985). Simultaneous response of myocardial contractility and a major proteolytic process to β-adrenergic receptor occupancy in the Langendorff isolated perfused rat heart. Biochem. J. **231,** 299–308.

LOCKWOOD, T.D. (1988). Distinctions between major chloroquine-inhibitable and adrenergic-responsive pathways of protein degradation and their relation to tissue ATP content in the Langendorff isolated perfused rat heart. Biochem. J. **251,** 341–346.

LOCKWOOD, T.D. (1997). Inactivation of intracellular proteolysis and cathepsin B enzyme activity by dehydroascorbic acid and reactivation by dithiothreitol in perfused heart. Biochem. Pharmacol. **54,** 669–675.

LOCKWOOD, T.D. (1999). Redox-dependent and redox-independent subcomponents of protein degradation in perfused myocardium. Am. J. Physiol. **276,** 945–954.

LOCKWOOD, T.D. (2000). Characterization of proteolytic pathways with antiparasitic drugs in the perfused rat heart (manuscript in preparation).

LOECHLER, EL.L., and HOLLOCHER, T.C. (1975). Mechanism of the reaction of dithiols with flavins. J. Am. Chem. Soc. **97,** 3235–3237.

LUIKENHUIS, S., PERRONE, G., DAWES, I.W., and GRANT, C.M. (1998). The yeast Saccharomyces cerevisiae contains two glutaredoxin genes that are required for protection against reactive oxygen species. Mol. Biol. Cell. **9,** 1081–1091.

LUNDSTROM-LJUNG, J., VLAMIS-GRADIKAS, A., ASLUND, F., and HOLMGREN, A. (1999). Reactivity of glutaredoxins 1, 2 and 3 from Escherichia coli and protein disulfide isomerase towards glutathionyl-mixed disulfides in ribonuclease A. FEBS Lett. **443,** 85–88.

MAHMOOD, N., JHAUMEER, LUALO, S., SAMPSON, J., and HOUGHTON, P.J. (1998). Anti-HIV activity and mechanism of action of macrocyclic diamide SRR-SB3. J. Pharm. Pharmacol. **50,** 1339–1342.

MARTENSSON, J., and MEISTER, A. (1991). Glutathione deficiency decreases tissue ascorbate levels in newborn rats: Ascorbate spares glutathione and protects. Proc. Natl. Acad. Sci. USA **88,** 4656–4660.

MAY, J.M., QU, Z.C., and WHITESELL, R.R. (1995). Ascorbate is the major electron donor for a transmembrane oxidoreductase of human erythrocytes. Biochem. Biophys. Acta **1238,** 127–136.

MAY, J.M., MENDIRATTA, S., HILL, K.R., and BURK, R.F. (1997). Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. J. Biol. Chem. **272,** 22607–22610.

MAY, J.M., COBB, C.E., MENDIRATTA, S., HILL, K.E., and BURK, R.F. (1998). Reduction of the ascorbyl free radical to ascorbate by thioredoxin reductase. J Biol. Chem. **273,** 23039–23045.

McKERROW, J. (1999). Development of cysteine protease inhibitors as chemotherapy for parasitic diseases: insights on safety, target validation and mechanism of action. Int. J. Parasitol. **29,** 833–837.

McKERROW, J.H., SUN, E., ROSENTHAL, P.J., and BOUVIER, J. (1993). The proteases and pathogenicity of parasitic protozoa. Annu. Rev. Microbiol. **47,** 821–853.

MEHTA, S., VELMURUGAN, S., and LOBO, Z. (1998). Repression of enzymes of the pentose phosphate pathway by glucose in fission yeast. FEBS Lett. **440,** 430–433.

MEISTER, A. (1992). On the antioxidant effects of ascorbic acid and glutathione. Biochem. Pharmacol. **44,** 1905–1915.

MEISTER, A. (1994). Glutathione-ascorbic acid antioxidant system in animals. J. Biol. Chem. **269,** 9397–9400.

MEISTER, A. (1995). Glutathione metabolism. Methods Enzymol. **251,** 3–7.

MENDIRATTA, S., QUZ, C., and MAY, J.M. (1998a). Erythrocyte ascorbate recycling: antioxidant effects in blood. Free Radic. Biol. Med. **24,** 789–797.

MENDIRATTA, S., QU, Z.C., and MAY, J.M. (1998b). Enzyme-dependent ascorbate recycling in human erythrocytes: Role of thioredoxin reductase. Free Rad. Biol. Med. **25,** 221–228.

MILLER, R.M., PARK, E.M., and THOMAS, J.A. (1991). Reduction (dethiolation) of protein-mixed disulfides; Distribution and specificity of dethiolating enzymes and N,N′-bis (2-chloroethyl)-N-nitrosourea inhibition of an NADPH-dependent cardiac dethiolase. Arch Biochem. Biophys. **287,** 112–120.

MIZUSHIMA, N., NODA, T., YOSHIMURI, T., TANAKA, Y., ISHII, T., GEORGE, M.D., KLIONSKI, D.J., and OSHUMI, M. (1998). A protein conjugation system of autophagy. Nature **395,** 395–398.

MORELL, S., FOLLMANN, H., DE TULLIO M., and HABERLEIN, I. (1997). Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants. FEBS Lett. **414,** 567–570.

MORGAN, H.E. (1982). Protein degradation in the energy-poor heart. In *Congestive Heart Failure: Current research and clinical applications*. E. Braunwald, ed. (Grune & Stratton, Orlando, FL) pp. 43–49.

MOSSNER, E., HUBER, WUNDERLICH, M., RIETCH, A., BECKWITH, J., GLOCKSHUBER, R., and ASLUND, F. (1999). Importance of redox potential for the *in vivo* function of the cytoplasmic disulfide reductant thioredoxin from Escherichia coli. J. Biol. Chem. **274,** 25254–25259.

MULLER, E.G. (1996). A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth. Mol. Biol. Cell. **7,** 1805–1813.

MULLER, S., GILBERGER, T.W., FARBER, P.M., BECKER, K., SCHIRMER, R.H., and WALTER, R.D. (1996). Recombinant putative glutathione reductase of Plasmodium falciparum exhibits thioredoxin reductase activity. Mol. Biochem. Parasitol. **80,** 215–219.

NAKAMURA, H., NAKAMURA, K., and YODOI, J. (1997). Redox regulation of cellular activation. Annu. Rev. Immunol. **15,** 351–369.

NORDBERG, J., ZHONG, L., HOLMGREN, A., and AMES, E.S. (1998). Mammalian thioredoxin reductase is irreversibly inhibited by dinitrohalobenzenes by alkylation of both the redox active selenocysteine and its neighboring cysteine residue. J. Biol. Chem. **273,** 10835–10842.

NORDSTRAND, K., SLUND, F., HOLMGREN, A., OTTING, G., and BERNDT, K.D. (1999). NMR structure of Escherichia coli glutaredoxin 3-glutathione mixed disulfide complex: implications for the enzymatic mechanism. J. Mol. Biol. **286,** 541–552.

OBIN, M., SHANG, F., GONG, S., HANDELMAN, G., BLUMBERG, J., and TAYLOR, A. (1998). Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide. FASEB J. **12,** 561–569.

OLIVEIRA, L., BOUTON, C., and DRAPIER, J.C. (1999). Thioredoxin activation of iron regulatory proteins. Redox regulation of RNA binding after exposure to nitric oxide. J. Biol. Chem. **274,** 516–521.

PACKER, L., WITT, E.H., and TRITSCHLER, H.J. (1995). Alpha-Lipoic acid as a biological antioxidant. Free Radic. Biol. Med. **19,** 227–250.

PAGET, M.S., KANG, J.G., ROE, J.H., and BETTNER, M.J. (1998). SigmaR, an RNA polymerase sigma factor that modulates expression of the thioredoxin system in response to oxidative stress in Streptomyces coilicolar A3(2). EMBO. J. **17,** 5776–5782.

PARK, J.B., and LEVINE, M. (1996). Purification, cloning and expression of dehydroascorbic acid-reducing activity from human neutrophils: identification as glutaredoxin. Biochem. J. **315,** 931–938.

PEDRAJAS, J.R., KOSMIDOU, E., MIRANDA-VIZUETE, A., GUSTAFSSON, J.A., WRIGHT, A.P., and SPYROU, G. (1999). Identification and functional characterization of novel mitochondrial thioredoxin system in Saccharomyces cerevisiae. J. Biol. Chem. **274,** 6366–6373.

PERLER, F.B., XU, M., and PAULUS, H. (1997). Protein splicing and autoproteolysis mechanisms. Curr. Opin. Chem. Biol. **1,** 292–299.

PRINZ, W.A., ASLUND, F., HOLMGREN, A., and BECKWITH, J. (1997). The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. J. Biol. Chem. **272,** 15661–15667.

RAMAKRISHNA, RAO, D.N., FISCHER, V., and MASON, R.P. (1990). Glutathione and ascorbate reduction of the acetaminophen radical formed by peroxidase. Detection of the glutathione disulfide radical anion and the ascorbyl radical. J. Biol. Chem. **265,** 844–847.

REYES, J.C. (1996). Zinc transport in mammalian cells. Am. J. Physiol. **270,** C401–C410.

RIETSCH, A., BESSETTE, P., GEORGIOU, G., and BECKWITH, J. (1997). Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. J. Bacteriol. **179,** 6602–6608.

RODRIQUEZ, E., MANZANEQUE, M.T., ROS, J., CABISCOL, E., SORRIBAS, A., and HERRERO, E. (1999). Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in Saccharomyces cerevisiae. Mol. Cell. Biol. **19,** L180–L190.

ROSE, R.C., and BODE, A.M. (1992). Tissue-mediated regeneration of ascorbic acid: Is the process enzymatic? Enzyme **46,** 196–203.

ROSENTHAL, P.J., LEE, G.K., and SMITH, R.E. (1993). Inhibition of a plasmodium vinkei cysteine proteinase cures murine malaria. J. Clin. Investig. **91,** 1052–1056.

ROSSIG, L., FICHTLSCHERER, B., BREITSCHOPF, K., HAENDELER, J., ZEIHER, A.M., MULSCH, A., and DIMMELER, S. (1999). Nitric oxide inhibits caspase-3 by s-nitrosation in vivo. J. Biol. Chem. **274,** 823–826.

ROY, S., and PACKER, L. (1998). Redox regulation of cell functions by alpha-lipoate: biochemical and molecular aspects. Biofactors **8,** 17–21.

RUSAKOW, L.S., HAN, J., HAYWARD, M.A., and GRIFFITH, O.W. (1995). Pulmonary oxygen toxicity in mice is characterized by alterations in ascorbate redox status. J. Appl. Physiol. **79,** 1769–1776.

SALREMINI, F., FRONZE, A., IERVOLENO, A., FILOSA,

S., SOLZANO, S., and URSINI, M.V. (1999). Enhanced glutathione levels and oxidoresistance mediated by increased glucose-6-phosphate dehydrogenase expression. J. Biol. Chem. **274,** 2750–2757.

SASODA, T., NAKAMURA, H., UEDA, S., SADO, N., KITAOK, Y., TAKABAYASHI, A., SPYROU, G., HOLMGREN, A., and YODOI, J. (1999). Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum (II). Free Radic. Biol. Med. **27,** 504–514.

SATRUSTEGUI, A.A., and MACHADO, A. (1980). Development of NADPH-producing pathways in rat heart. Biochem. J. **18,** 799–803.

SCHOLZ, T.D., LAUGHLIN, M.R., BALABAN, R.S., KUPRIYANOV, V.V., and HEINEMAN, F.W. (1995). Effect of substrate on mitochondrial NADH, cytosolic redox state, and phosphorylated compounds in isolated hearts. Am. J. Physiol. **268,** H82–H91.

SEHAJPAL, P.K., BASU, A., OGISTE, J.S., and LANDER, H.M. (1999). Reversible S-nitrosation and inhibition of HIV-1 protease. Biochemistry **38,** 407–413.

SEN, C.K. (1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. Biochem. Pharmacol. **55,** 1747–1758.

SEN, C.K. (1999). Glutathione homeostasis in response to exercise training and nutritional supplements. Mol. Cell Biochem. **196,** 31–42.

SEN, C.K., and PACKER, L. (1996). Antioxidants and redox regulation of gene transcription. FASEB J. **10,** 709–720.

SEN, C.K., ROY, S., and PACKER, L. (1999). Fas-mediated apoptosis of human Jurkat T-cells: Intracellular events and potentiation by redox-active α-lipoic acid. Cell Death Different. **6,** 481–491.

SHANG, F., GONG, X., and TAYLOR, A. (1997). Activity of ubiquitin-dependent pathway in response to oxidative stress. Ubiquitin-activating enzyme is transiently up-regulated. J. Biol. Chem. **272,** 23086–23093.

SHI, J., VLAMIS-GARDIKAS, A., ASLUND, F., HOLMGREN, A., and ROSEN, B.P. (1999). Reactivity of glutaredoxin 1,2 and 3 from Escherichia coli shows that glutaredoxin 2 is the primary hydrogen donor to Ars C-catalyzed arsenate reduction. J. Biol. Chem. **274,** 36039–36042.

SHOFFNER, J.M. (1997). Oxidative phosphorylation defects and Alzheimer's disease. Neurogenetics **1,** 13–19.

SIES, H. (1997). Oxidative stress: oxidants and antioxidants. Exp. Physiol. **82,** 291–295.

SIES, H., DAFRE, A.L., JI, Y., and AKERBOOM, T.P. (1999). Protein S-thiolation and redox regulation of membrane-bound glutathione transferase. Chem. Biol. Int. **111,** 177–185.

SIGEL, H., PRIJS, B., McCORMICK, D.B., and SHIH, J.C. (1978). Stability and structure of binary and ternary complexes of alpha-lipoate and lipoate derivatives with Mn^{2+}, Cu^{2+}, and Zn^{2+} in solution. Arch. Biochem. Biophys. **187,** 208–214.

SIMONIAN, N.A., and COYLE, J.T. (1996). Oxidative stress in neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol. **37,** 83–106.

SINGH, R., BLATLER, W.A., and COLLISON, A.R. (1995). Thiol assay based upon reactivation of papain. Methods Enzymol. **251,** 229–237.

SLEKAR, K.H., KOSMAN, D.J., and CULOTTA, V.C. (1996). The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. J. Biol. Chem. **271,** 28831–28836.

SLUSAREWICZ, P., XU, Z., SEEFELD, K., HAAS, A., and WICKNER, W.T. (1997). 12B is a small cystolic protein that participates in vacuole fusion. Proc. Natl. Acad. Sci. USA **94,** 5582–5587.

SPYROU, G., and HOLMGREN, A. (1996). Deoxyribonucleoside triphosphate pools and growth of glutathione-depleted 3T6 mouse fibroblasts. Biochem. Biophys. Res. Commun. **220,** 42–46.

STARKE, D.W., CHEN, Y., BAPNA, C.P., LESNEFSKY, E.J., and MIEYAL, J.J. (1997). Sensitivity of protein sulfhydryl repair enzymes to oxidative stress. Free Radic. Biol. Med. **23,** 373–384.

STATHAKIS, P., FITZGERALD, M., MATTHIAS, L.J., CHESTERMAN, C.N., and HOGG, P.J. (1997). Generation of angiostatin by reduction and proteolysis of plasmin: catalysis by a plasmin reductase secreted by cultured cells. J. Biol. Chem. **272,** 20641–20645.

STEPHEN, A.G., POWLS, R., and BEYNON, R.L. (1993). Activation of oxidized cysteine proteinases by thioredoxin-mediated reduction *in vitro*. Biochem. J. **291,** 345–347.

STEWART, E.J., ASLUND, F., and BECKWITH, J. (1998). Disulfide bond formation in the Escherichia coli cytoplasm: An in vivo role reversal for the thioredoxins. EMBO. J. **17,** 5543–5550.

STORER, A.C., and MENARD, R. (1994). Catalytic mechanism in papain family of cysteine peptidases. Methods Enzymol. **244,** 486–500.

STUBAUER, G., GIUFFRE, A., and SARTI, P. (1999). Mechanism of S-nitrosothiol formation and degradation mediated by ions. J Biol. Chem. **274,** 128–133.

SUZUKI, D.K., REP, M., MAARTEN VAN DIJL, J., SUDO, K., GRIRELL, L.A., and SCHATZ, G. (1997). ATP dependent proteases that also chaperone protein biogenesis. Trends Biochem. Sci. **22,** 118–123.

THORNE, D.P., and LOCKWOOD, T.D. (1991). Effect of Zn^{2+} on the proteolytic inhibitory action of insulin and biguanide antihyperglycemic drugs. Diabetes **40,** 612–620.

THORNE, D.P., and LOCKWOOD, T.D. (1993). Distinctions between four major proteolytic processes of myocardium, one insensitive to thiol-reactive agents and thiol protease inhibitor. Am. J. Physiol. **265,** E10–E19.

TORBRELLA, D., and PLOEGH, H.L. (1998). Dislocation of type I membrane proteins from the ER to the cytosol is sensitive to changes in redox potential. J. Cell Biol. **142,** 365–376.

URSINI, M.V., PARRELLA, A., ROSA, G., SALZANO, S., and MARTINI, G. (1997). Enhanced expression of glucose-6-phosphate dehydrogenase in human cells sustaining oxidative stress. Biochem. J. **323,** 801–806.

VASQUEZ-VIVAR, J., and AUGUSTO, O. (1992). Hy-

droxylated metabolites of the antimalarial drug primaquine. Oxidation and redox cycling. J. Biol. Chem. **267,** 6848–6854.

VERA, J.C., RIVAS, C.I., FISCHBORG, J., and GOLDE, D.W. (1993). Mammalian facilitative hexose transporters mediate the transport of dehydroascorbic acid. Nature **364,** 79–82.

VERBUNT, R.J., and VAN DER LAARSE, A. (1997). Glutathione metabolism in non-ischemic and post-ischemic rat hearts in response to an exogenous pro-oxidant. Mol. Cell. Biochem. **167,** 127–134.

VETHANAYAGAM, J.G., GREEN, E.H., ROSE, R.C., and BODE, A.M. (1999). Glutathione-dependent ascorbate recycling activity of rat serum albumin. Free Radic. Biol. Med. **26,** 1591–1598.

VINA, J., SASTRE, J., ASENI, M., and PACKER, L. (1995). Assay of blood glutathione oxidation during physical exercise. Methods Enzymol. **251,** 237–243.

VULPE, C.D., and PACKMAN, S. (1995). Cellular copper transport. Annu. Rev. Nutri. **15,** 293–322.

WAGLE, A., JIVRAJ, S., GARLOCK, G.L., and STAPLETON, S.R. (1998). Insulin regulation of glucose-6-phosphate dehydrogenase gene expression is rapamycin-sensitive and requires phosphatidylinositol 3-kinase. J. Biol. Chem. **273,** 14968–14974.

WANG, P.F., ARSCOTT, L.D., GILBERGER, T.W., MULLER, S., and WILLIAMS, C.H., JR. (1999). Thioredoxin reductase from Plasmodium falciparum: evidence for interaction between the C-terminal cysteine residues and the active site disulfide-dithiol. Biochemistry **38,** 3187–3196.

WASHBURN, M.P., and WELLS, W.W. (1997). Glutathione dependent reduction of alloxan to dialuric acid catalyzed by thioltransferase (glutaredoxin): A possible role for thioltransferase in alloxan toxicity. Free Radic. Biol. Med. **23,** 563–570.

WASHBURN, M.P., and WELLS, W.W. (1999a). The catalytic mechanism of the glutathione-dependent dehydroascorbate reductase activity of thioltransferase (glutaredoxin). Biochemistry **38,** 268–274.

WASHBURN, M.P., and WELLS, W.W. (1999b). Identification of the dehydroascorbic acid reductase and thioltransferase (Glutaredoxin) activities of bovine erythrocyte glutathione peroxidase. Biochem. Biophys. Res. Commun. **257,** 567–571.

WASHKO, P.W., WELCH, R.W., DHARIWAL, K.R., WANG, Y., and LEVINE, M. (1992). Ascorbic acid and dehydroascorbic acid analysis in biological samples. Anal. Biochem. **204,** 1–14.

WELCH, R.W., WANG, Y., CROSSMAN, A., PARK, J.B., KIRK, K.L., and LEVINE, M. (1995). Accumulation of vitamin C (ascorbate) and its oxidized metabolite dehydroascorbic acid occurs by separate mechanisms. J. Biol. Chem. **270,** 12584–12592.

WELLS, W.W., XU, D.P., YANG, Y., and ROCQUE, P.A. (1990). Mammalian thiol transferase (Glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. J. Biol. Chem. **265,** 15361–15364.

WINKER, B.S. (1992). Unequivocal evidence in support of the nonenzymatic redox coupling between glutathione/glutathione disulfide and dehydroascorbic acid. Biochim. Biophys. Acta **1117,** 287–290.

WINTERBOURNE, C.C., and MUNDAY, R. (1989). Glutathione-mediated redox cycling of alloxan. Biochem. Pharmacol. **38,** 271–277.

WONG, J.H., KOBREHEL, K., and BUCHANAN, B.B. (1995). Thioredoxin and seed proteins. Methods Enzym. **252,** 228–240.

WYNN, R., COCCO, M.J., and RICHARDS, F.M. (1995). Mixed disulfide intermediates during the reduction of disulfides by Escherichia coli thioredoxin. Biochemistry **34,** 11807–11813.

XU, Z., and WICKNER, W. (1996). Thioredoxin is required for vacuole inheritance in Saccharomyces cerevisiae. J. Cell Biol. **132,** 787–794.

YAKULIS, V., SCHMALE, J., COSTEA, N., and HELLER, P. (1968). Production of Fc Fragments of IgM. J. Immunol. **100,** 525–529.

YANG, Y., JAO, S.C., NANDURI, S., STARKE, D.W., MIEVAL, J.J., and QIN, J. (1988). Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity. Biochemistry **37,** 17145–17156.

YOUNG, J., KANE, L.P., EXLEY, M., and WILEMAN, T. (1993). Regulation of selective protein degradation in the endoplasmic reticulum by redox potential. J. Biol. Chem. **268,** 19810–19818.

ZHONG, L., ARNER, E.S., LJUNG, J., ASLUND, F., and HOLMGREN, A. (1998). Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. J. Biol. Chem. **273,** 8581–8591.

ZIEGLER, D.M. (1985). Role of reversible oxidation of enzyme thiols-disulfides in metabolic regulation. Annu. Rev. Biochem. **54,** 305–329.

ZIMMER, H.G. (1996). Regulation of and intervention into the oxidative pentose phosphate pathway and adenine nucleotide metabolism in the heart. Mol. Cell Biochem. **160–161,** 101–109.

Address reprint requests to:
Thomas D. Lockwood
Department of Pharmacology
School of Medicine
Wright State University
Dayton, OH 45435

Fax: 937-775-7221

Received for publication February 7, 2000

This article has been cited by:

1. Joanna M Rybicka, Dale R Balce, Sibapriya Chaudhuri, Euan R O Allan, Robin M Yates. 2011. Phagosomal proteolysis in dendritic cells is modulated by NADPH oxidase in a pH-independent manner. *The EMBO Journal* . [CrossRef]
2. Byron K.Y. Bitanihirwe, Tsung-Ung W. Woo. 2011. Oxidative stress in schizophrenia: An integrated approach. *Neuroscience & Biobehavioral Reviews* **35**:3, 878-893. [CrossRef]
3. Mario Menschikowski, Albert Hagelgans, Graeme Eisenhofer, Oliver Tiebel, Gabriele Siegert. 2010. Reducing agents induce thrombomodulin shedding in human endothelial cells. *Thrombosis Research* **126**:2, e88-e93. [CrossRef]
4. J. M. Rybicka, D. R. Balce, M. F. Khan, R. M. Krohn, R. M. Yates. 2010. NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the lumenal redox environment of phagosomes. *Proceedings of the National Academy of Sciences* **107**:23, 10496-10501. [CrossRef]
5. Mario C. De Tullio. 2010. Antioxidants and redox regulation: Changing notions in a changing world. *Plant Physiology and Biochemistry* **48**:5, 289-291. [CrossRef]
6. M.M. Elahi, B.M. Matata. 2006. Free radicals in blood: Evolving concepts in the mechanism of ischemic heart disease. *Archives of Biochemistry and Biophysics* **450**:1, 78-88. [CrossRef]
7. Jaanus Kruusma, Adam M. Benham, J. A. Gareth Williams, Ritu Kataky. 2006. An introduction to thiol redox proteins in the endoplasmic reticulum and a review of current electrochemical methods of detection of thiols. *The Analyst* **131**:4, 459. [CrossRef]
8. V ANDELA, E SCHWARZ, R OKEEFE, E PUZAS, J ROSENBLATT, R ROSIER. 2005. A genome-wide expression profile and system-level integration of nuclear factor kappa B regulated genes reveals fundamental metabolic adaptations during cell growth and survival. *FEBS Letters* **579**:30, 6814-6820. [CrossRef]
9. Frank J. Giordano. 2005. Oxygen, oxidative stress, hypoxia, and heart failure. *Journal of Clinical Investigation* **115**:3, 500-508. [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]
11. John J Haddad. 2002. Pharmaco-redox regulation of cytokine-related pathways: from receptor signaling to pharmacogenomics. *Free Radical Biology and Medicine* **33**:7, 907-926. [CrossRef]
12. Thomas D. Lockwood . 2002. Cathepsin B Responsiveness to Glutathione and Lipoic Acid Redox. *Antioxidants & Redox Signaling* **4**:4, 681-691. [Abstract] [Full Text PDF] [Full Text PDF with Links]