Pharmaceutical Research, Vol. 4, No. 4, 1987

Review

Protein Degradation: The Role of Mixed-Function Oxidases

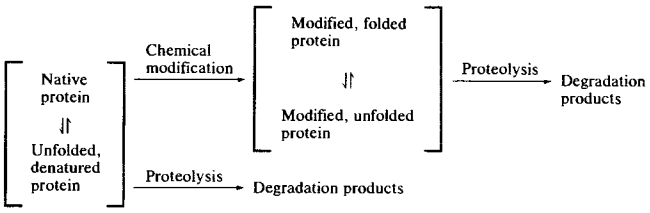
Valerie Daggett[1]

The mechanisms by which protein oxidation is mediated in the cell are of both biological and pharmacological importance. Oxidases responsible for the metabolism of xenobiotics catalyze the oxidative inactivation of select enzymes. Oxidation mediated by mixed-function oxidase (MFO) systems renders proteins more susceptible to proteolysis and, consequently, appears to be a signal for protein degradation. The mode of action of MFO systems is discussed in detail for a specific, well-characterized system—the MFO-catalyzed oxidation of glutamine synthetase (GS). Findings for this system are then generalized to help explain how other metabolic enzymes are oxidized by MFO systems. The broader consequences of oxidative mechanisms are discussed. For example, the accumulation of modified proteins during aging and in some premature aging diseases may be due in part to shifts in the relative rates of oxidation and degradation for these proteins. Further, the oxidation of key metabolic enzymes appears to be responsible for the bacteriocidal action of neutrophils. There is also some evidence that the degradation of endogenous proteins increases following ingestion of, or exposure to, agents that induce MFO activity.

KEY WORDS: protein degradation; mixed-function oxidase system; aging; chemical modification.

I. INTRODUCTION

Herein we examine the pathways of protein degradation and discuss in detail those that involve protein oxidation as the initial signal for degradation. Protein degradation protects a cell against the toxic accumulation of abnormal proteins and proteins that are no longer necessary. This is of pharmacological relevance because of the increasing number of potential protein drugs. Some of the protein drugs produced by genetic engineering and hybridoma technology are contained in Table I. (See Ref. 1 for a more comprehensive list.)



Scheme I

Scheme I depicts the general pathways that are thought to be involved in protein degradation. The equilibrium between folded and unfolded protein affects the proteolytic cleavage of these molecules to degradation products. Many chemical modifications of proteins that facilitate proteolysis have been reported. The degradation rate for a modified protein is generally greater than that for the corresponding unaltered, native protein. Chemical modification may alter the degradation rate by modulating the unfolding equilibrium. (These relationships are discussed in Section II.)

Most of the available experimental data relate to intracellular protein degradation. However, while intracellular processes are important, in the case of protein drugs extracellular degradation will also be an important determinant to reaching the target site. It has been suggested that activation of plasminogen by tissue plasminogen activator (TPA) provides the general mechanism for localized extracellular proteolysis (2). The administration of TPA leads to the dissolution of blood clots *in vivo*, demonstrating a beneficial effect of proteolysis with regard to protein drugs. It appears that a limited number of cell surface peptidases is responsible for hydrolyzing small biologically active polypeptides in all tissues and organs. So, unlike the presumably specific interactions between a peptide and its receptor, the inactivation by peptidases is relatively nonspecific (3). There is evidence that endopeptidase-24.11 is involved in the inactivation of some neuropeptides in the brain (3). Although little work has been done in this area, it is conceivable that regulatory peptides in the immune and endocrine systems may be inactivated in a similar manner, which can obviously affect the therapeutic efficacy of such drugs.

The emphasis here is on factors that influence intracellular protein degradation. Mixed-function oxidase-catalyzed oxidation of proteins is an example of a chemical modification (as depicted in Scheme I), or "marking step," that facil-

[1] Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143.

0724-8741/87/0800-0278$05.00/0

Table I. Some Protein Drugs Produced by Genetic Engineering

Epidermal growth factor
Hepatitis B vaccine
Human growth hormone
Human insulin
Interferons (α, β, γ)
Interleukin (IL-2)
Malaria vaccine
Retrovirus vaccine
Tissue plasminogen activator (TPA)
Tumor necrosis factor (TNF)

itates proteolysis. The physiological significance of oxidation as a signal for protein degradation includes the accumulation of modified proteins during aging and in some premature aging diseases. Further, the enzymatic oxidation of key metabolic enzymes may be responsible for the bactericidal action of neutrophils.

II. PROTEIN DEGRADATION AND ITS RELATION TO PROTEIN STRUCTURE

Intracellular protein breakdown protects the cell against the toxic accumulation of abnormal polypeptides arising because of missense mutations, mistakes in RNA or protein synthesis, incorporation of amino acid analogues, posttranslational modifications, or intracellular denaturation. Orgel (4) has suggested that the accidental production of abnormal proteins will result in the accumulation of these erroneous polypeptides. However, under normal conditions, proteins that have been chemically modified, mutated, or otherwise altered are almost invariably degraded very rapidly in the cell (5).

A folded, native protein residues in a local minimum energy conformation. The energy difference between a protein in the native and unfolded states is typically 5 to 15 kcal/mol, allowing the forms to interconvert quite rapidly (on the millisecond–second time scale) (6). Studies suggest that the equilibrium between folded and unfolded states may be more important in determining proteolytic susceptibility than the native protein conformation per se (6,7). Therefore, the overall rate of degradation might be governed in part by the equilibrium concentration of unfolded (or partially unfolded) forms of the protein, assuming that the unfolded form is the substrate for proteolysis.

Rates of protein degradation *in vivo* vary greatly. Those proteins degraded most rapidly in the cell generally catalyze rate-determining steps of metabolic reactions (8), such that their levels regulate the flux of metabolites. Correlations between protein structure and degradation rates have been sought to explain the basis of protein turnover. Degradation rates have been correlated with thermal stability (9), dissociation of stabilizing ligands (10), and susceptibility to proteolytic cleavage (11). These observations are consistent with the idea that an initial proteolytic cleavage is the rate-determining step in protein degradation and that cleavage is most likely to occur in a transiently unfolded or partially unfolded state. Therefore, an increase in the amount of nonnative protein present might lead to increased degradation. A recent finding suggesting that the half-life of a protein is a function of its amino-terminal residue (the N-end rule) (12) may also be consistent with the contention that protein cleavage is the rate determining step in protein degradation. Bachmair *et al.* (12) suggest that the rate-determining step in the degradation of long-lived proteins is slow aminopeptidase cleavage which exposes a destabilizing amino acid. The destabilizing element is rapidly recognized and leads to degradation according to the N-end rule.

Other observations are not consistent with a simple proteolytic mechanism of degradation. For example, acidic proteins are generally degraded more rapidly than are neutral or basic ones (13), the rate of degradation is nearly proportional to the amount of apolar surface area of the folded protein (8), and proteins composed of large polypeptide chains are degraded more rapidly than those composed of small chains (14). A recent literature search revealed that proteins that are rapidly degraded in eukaryotic cells contain regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T)—the PEST hypothesis (15). This hypothesis appears to be consistent with observations that acidic proteins are generally degraded more rapidly than basic proteins, as PEST proteins tend to be acidic. In view of conflicting observations regarding the determinants of protein half-life, there is no reason to assume that the mechanism of protein degradation is the same for every protein or that the initial rate-determining step is necessarily proteolytic cleavage. However, given what is now known of the factors that affect protein degradation and the increasing interest in the use of polypeptides as drugs or as carriers for drugs, it may be possible to design proteins with controlled lifetimes by, for example, attaching PEST sequences.

A. Chemical Modification

If the rate of protein degradation is governed in part by the equilibrium concentration of unfolded protein, then increases and decreases in degradation rates may be due to factors that modulate the unfolding equilibrium. Chemical modification may shift the distribution toward a higher concentration of unfolded species, thereby explaining the facile degradation *in vivo*. At least nine such chemical modifications, "marking" steps, that facilitate proteolytic attack are known. These are listed in Table II. One such chemical modification, the oxidation of amino acids by mixed-function oxidase (MFO) systems, is the primary focus of this paper.

Table II. Chemical Modifications of Proteins that Facilitate Proteolysis

Chemical modification	Ref. No.
Phosphorylation of serine and threonine residues	16
Formation of mixed disulfide derivatives of cysteine residues	17
Carbamylation of lysine residues	18
Oxidation of nonheme iron sulfur centers	19
Conjugation of ϵ-amino groups of lysine with ubiquitin	20
Oxidation of methionine to methionine sulfoxide and methionine sulfone	21
Deamidation of glutamine and asparagine residues	22
Glycosylation	23
Oxidation of amino acids by MFO systems	24

B. Substrate Availability

Substrate availability can also modulate the unfolding equilibrium. Bound substrate generally protects proteins from degradation. Thus, substrate binding may shift the distribution toward a higher concentration of folded species. Variations in substrate concentrations accompanying shifts in the nutritional state of an organism could partially account for the diversity of turnover rates for different enzymes and for variations in the rates of degradation of individual enzymes (24).

Substrate availability modulates the susceptibility of glutamine synthetase (GS) to oxidative inactivation. GS is adenylylated in its inactive form; the unadenylylated form is the physiologically functional state (25). In the absence of substrates, adenylylated GS is no more susceptible to oxidation than the unadenylylated forms (26). Adenylylation of native GS has no effect on susceptibility to degradation. In the presence of one of three substrates—ammonia, glutamate, or ATP—the inactivation of adenylylated GS by oxidation is only slightly stimulated (27). If both ATP and glutamate are present at physiological concentrations, the unadenylylated form is protected against oxidation; however, oxidation of the adenylylated form increases fivefold under the same conditions (27). The effect is directly proportional to the extent of adenylylation. This dependence on adenylylation has been found in every MFO system studied and appears to be important not only in metabolic regulation but perhaps also in degradative regulation.

These results suggest that when there is a demand for glutamine, glutamine synthetase is in its unadenylylated state and protected against MFO systems. In the presence of excess glutamine the enzyme exists in its adenylylated state and can be readily oxidized. GS becomes susceptible to oxidation under either of two conditions (27): (i) substrates are not available, or (ii) cellular requirements for glutamine have been met (in which case GS is adenylated and inactive).

Two other enzymes that are oxidized by MFO systems have also been shown to be protected against oxidation by the presence of their substrates. Phosphoenolpyruvate and ATP decrease the susceptibility of pyruvate kinase to oxidation, and ATP and 3-phosphoglycerate protect phosphoglycerate kinase (all at physiological concentrations) (28).

III. MIXED-FUNCTION OXIDASE-CATALYZED OXIDATION OF PROTEINS

One of the critical pathways in the metabolism of many xenobiotics, toxins, and carcinogens involves the two-electron reductive cleavage of molecular oxygen with concomitant substrate hydroxylation as catalyzed by MFO systems. Many enzymatic oxidations conform to the scheme below (where M is a transition metal, typically copper or iron) (29).

$$H_2O_2 \rightleftharpoons \begin{matrix} O_2 \\ O_2^{\cdot -} \\ HO^{\cdot} \\ H_2O \end{matrix} \begin{matrix} \diagdown \\ \diagdown \\ \diagdown \\ \diagdown \end{matrix} \begin{matrix} \text{Substrate} \\ M^{n+} \\ \\ \text{Oxidized product} \end{matrix}$$

The single-electron reduction of oxygen yields superoxide ($O_2^{\cdot -}$), which may either disproportionate or accept another electron from a reducing agent to yield hydrogen peroxide (H_2O_2). Hydrogen peroxide and superoxide are directly damaging to cellular components. Further, they may interact to form a more reactive species—the hydroxyl radical ($OH^{\cdot}$). The production of $OH^{\cdot}$ via an iron-catalyzed reaction of superoxide and hydrogen peroxide proceeds according to a Haber–Weiss reaction, Eq. (1) (29).

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{3+}} O_2 + OH^{\cdot} + OH^- \qquad (1)$$

There is *in vitro* evidence supporting the production of hydroxyl radical via the reaction of hydrogen peroxide with chelates of copper and iron (e.g., iron/histidinyl complexes) in a Fenton-type reaction, Eq. (2) (30).

$$Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + Fe(III) + OH^- \qquad (2)$$

Hydroxyl radicals react rapidly with most molecules by hydrogen abstraction, addition, or electron transfer reactions (31).

Demonstration of radical formation *in vivo* is difficult; however, their existence is suggested by the presence of catalase and glutathione peroxidase (which decompose H_2O_2) and superoxide dismutase (which decomposes $O_2^{\cdot -}$). In fact, superoxide dismutase and glutathione peroxidase are the primary enzymatic antioxidants responsible for the maintenance of safe levels of activated oxygen *in vivo*. There is a variety of nonenzymatic antioxidants that also protect a cell against activated oxygen, including glutathione, histidine, uric acid, guanine, and vitamins A, C, and E. (For a more thorough discussion see Ref. 32.)

Mixed-function oxidase systems, both enzymatic and nonenzymatic, inactivate a variety of enzymes and render them more susceptible to proteolysis. The most thoroughly studied of these systems involves glutamine synthetase (GS) as the target for inactivation. The following discussion focuses on the mechanism of GS oxidation.

A. Oxidation of Glutamine Synthetase: *In Vitro* Studies

Glutamine synthetase has been oxidized by the following enzymatic systems: cytochrome P-450 (isozyme 2), P-450 reductase; NADH oxidase; xanthine oxidase; nicotinate hydroxylase; glucose oxidase; putidaredoxin reductase; putidaredoxin with or without P-450cam; and peroxidase (24). Glutamine synthetase is also inactivated by nonenzymatic systems comprised of ascorbate/molecular oxygen/Fe(III) and Fe(II)/molecular oxygen. All of these MFO model systems generate H_2O_2 and Fe(II). Fe(II) presumably binds to a metal binding site on GS and is subsequently oxidized by H_2O_2. This reaction leads to the site-specific generation of an activated oxygen species, which then oxidizes a nearby amino acid(s).

1. Enzymatic MFO Systems

The mechanism of oxidation of GS was examined using NADH oxidase and a reconstituted cytochrome P-450 system composed of P-450 (isozyme 2), P-450 reductase, and NADPH (28). The proposed mechanism is as follows.

(1) $NAD(P)H + O_2 + H+ \xrightarrow{MFO} H_2O_2 + NAD(P)+$

(2) $NAD(P)H + 2Fe(III) \xrightarrow{MFO} 2Fe(II) + NAD(P)+ + H+$

(3) $Fe(II) + H_2O_2 \longrightarrow$ activated oxygen + Fe(III)

(4) Activated oxygen + enzyme $\longrightarrow$ oxidized enzyme

Scheme II

The proposed role of hydrogen peroxide is supported by the finding that both catalase and peroxidase inhibit the inactivation of glutamine synthetase (26). No oxidation occurs if only H_2O_2 is present. Support for the involvement of Fe(III) comes from the observation that $FeCl_3$ stimulates production of the oxidized enzyme. The importance of Fe(II) is demonstrated by the fact that both NADH oxidase and the P-450 systems are capable of reducing Fe(III). Fe(II) and molecular oxygen catalyze the inactivation of glutamine synthetase (26), whereas Fe(III) and molecular oxygen do not (28), supporting reaction 2. Reaction 3 is supported by the result that Fe(II) and hydrogen peroxide together, but neither alone, catalyze the inactivation of glutamine synthetase. The reaction of Fe(II) and H_2O_2 generates activated oxygen species (e.g., $\cdot OH$, $O_2^{\cdot -}$, ^1O, etc.) (28).

The nature of the activated oxygen species has not been identified. Hydroxyl radical participation has been refuted by the failure of radical scavengers—mannitol, dimethyl sulfoxide, and thiourea—to inhibit the oxidation reaction of GS (28). Superoxide anion has also been ruled out, as superoxide dismutase does not inhibit the reaction (28). The reaction is inhibited by exogenous histidine, which is generally considered presumptive evidence for the role of singlet oxygen in oxidase catalyzed reactions (33). An alternative explanation is that histidine chelates necessary metal ions or exerts its effect through allosteric interaction with glutamine synthetase (27).

Even though free radical scavengers are ineffective in inhibiting GS oxidation, oxygen radical involvement cannot be ruled out. Because of the specific nature of the reaction, only one histidine per GS subunit is modified regardless of the MFO system employed, and reactions 2 and 3 in Scheme II probably occur at a metal binding site or at the catalytic site of GS. The generated radical may be inaccessible to scavengers.

Oxidative modification of glutamine synthetase by a system comprised of putidaredoxin reductase, putidaredoxin, Fe(III), and NADH is partially inhibited by superoxide dismutase, DMSO, or histidine (27). When this sytem is supplemented with P-450 (isozyme 2) the scavengers have little or no effect. The same is observed for a system containing xanthine oxidase, hypoxanthine, ferredoxin, and Fe(III) (34). These results suggest that the type of activated oxygen generated may be dependent on the MFO system employed.

2. *Nonenzymatic MFO Systems*

A nonenzymatic system composed of ascorbate and molecular oxygen has also been examined. Incubation of glutamine synthetase with ascorbate, O_2, and Fe(III) leads to rapid loss of catalytic activity (27), mimicking the characteristics of inactivation by the enzymatic MFO systems. The main function of ascorbate may be to generate Fe(II). This supposition is supported by the finding that $FeSO_4$, in the presence of molecular oxygen, is able to catalyze the oxidation of glutamine synthetase (27). Both the ascorbate and the ferrous sulfate systems have the same characteristics as the enzymatic MFO systems and therefore are good models for the oxidative inactivation of enzymes.

B. The Site of Oxidation in GS

One of the 16 histidine residues per GS subunit is modified by both enzymatic and nonenzymatic MFO systems (27). Loss of activity parallels loss of histidine. The structure of the modified histidine is still in question; however, it is known that the modified residue contains a carbonyl group (26). Photooxidation of free histidine gives aspartic acid and urea via several unstable intermediate compounds (35). In another enzyme system, generation of hydroxyl radical by copper/histidinyl complexes brings about the oxidation of histidine to asparate (36). The investigators who made this observation claim that the specificity of the attack at the histidine is due exclusively to the location of the metal at this residue.

The modified histidine of glutamine synthetase is essential for biosynthetic and glutamyl transferase activity (26), but this histidine need not lie at the active site. Catalytically active GS has divalent cations bound to several metal binding sites (37). Oxidation of a histidine may destroy one of these sites and prevent binding of the necessary cation. The oxidized histidine may provide a specific recognition site for proteases and/or render one or more peptide bonds labile to attack. Studies with small synthetic peptides (5–10 residues) have shown that oxidation of the imidazole ring of histidine renders the adjacent peptide bond more susceptible to cleavage (38).

C. Compartmentalization Studies

The physiological significance of oxidative modification by the P-450 system may be questioned, since P-450 is present in the microsomal membrane, whereas glutamine synthetase and the other enzymes observed to be inactivated are cytosolic. Such physical separation may hinder the effectiveness of the reaction, especially since the oxidation of drugs by the P-450-linked reactions is mediated through direct interaction of the oxidizable substrate with specific binding sites on the enzyme (39). Nakamura and coworkers (40) addressed this issue by examining the effects of compartmentalization on the oxidation of GS.

The oxidative inactivation of GS occurs in the presence of microsomes, suggesting that direct interaction between P-450 and GS is not necessary. When glutamine synthetase was partitioned from a purified, reconstituted P-450 system using a semipermeable memrane, inactivation decreased threefold. The reaction was inhibited when catalase was added to either of the two compartments. That significant inactivation occurred at all when glutamine synthetase and the P-450 system were separated shows that freely diffusible intermediates generated by the P-450 system are responsible for the oxidation of GS and that direct complexation is not required.

D. Other Enzymes Inactivated by MFO Systems

Although the discussion thus far has focused on glutamine synthetase, a variety of other enzymes has been shown to be inactivated by MFO systems. Seventeen of twenty-six enzymes examined have been found to be readily inactivated by MFO systems (28,41). Many of these enzymes require a divalent metal cation for activity and many possess a histidine residue at the catalytic site. However, while it has been found that the inactivation of glutamine synthetase, phosphoglycerate kinase (28), enolase (41), and superoxide dismutase (42) involves the oxidation of just one histidine residue, modification of other amino acids may be involved in the inactivation of the other enzymes tested. MFO-catalyzed oxidation is not indiscriminate; many enzymes are not inactivated by exposure to MFO systems (41).

Since inactivation of these enzymes has not been examined in detail, it is not known whether the activated oxygen species is the same in each case, but it is plausible that the process is governed by reaction 3 in Scheme II or an analogous reaction. Although these studies have focused on enzymes, other proteins should also be susceptible to oxidation by MFO systems, suggesting that this reaction may be important globally in regulation.

IV. INTRACELLULAR PROTEASES SPECIFIC FOR OXIDIZED GS

The discovery of four intracellular proteases that preferentially degrade the oxidized form of glutamine synthetase supports the role of oxidative modification in intracellular protein turnover (43). One of these is cathepsin D, which is lysosomal in origin. The other three are cytosolic enzymes —calpain I, calpain II, and alkaline protease.

The ratio of oxidized to native glutamine synthetase degraded by cathepsin D is pH dependent. At the pH optimum of cathepsin D, pH 3–4, there is a two- to threefold increase in the degradation of the oxidized form, but both forms of GS are denatured at low pH values. At pH values between 4 and 5 the oxidized form is degraded 10 times more rapidly than the native. This may be physiologically relevant since the pH inside of lysozomes is about 4.7 (44).

A mixture of calpain I and calpain II degrades oxidized glutamine synthetase 30 times faster than the native form. Native GS is not a substrate for these enzymes. Alkaline protease degrades oxidized glutamine synthetase 40- to 80-fold more rapidly than the native enzyme at pH 8.0. At higher pH values native GS is also degraded, which is presumably due to dissociation of the native dodecamer. Dissociation of the subunits with urea mitigates the distinction between oxidized and native GS by the proteases. This is consistent with an earlier finding that there are decreased subunit interactions in the oxidized form (45), which is probably an important determinant to proteolytic susceptibility.

Trypsin and papain-catalyzed proteolysis of GS has also been examined. A two- to four-fold increase in degradation of modified GS compared to the native conformation was observed for trypsin, while only a two-fold difference occurred with papain. Since oxidative modification can be catalyzed by several MFO systems present within the cell, it is noteworthy that there are proteases within mammalian cells that recognize the oxidized form of GS under *in vitro* conditions which mimic physiological states.

V. THE PHARMACOLOGICAL AND BIOCHEMICAL SIGNIFICANCE OF OXIDATION AS A MARKER FOR PROTEIN DEGRADATION

A. Aging

There are a number of proposed theories, both genetic and nongenetic, to explain the phenomenon of cellular aging (46). Each theory focuses on specific aspects of aging but none is capable of explaining all of the known facts. Among those theories that seem the most plausible are the codon restriction (47), error (48), and cross-linking theories (49).

The codon-restriction theory of aging suggests that as a result of differentiation, cells lose the ability to translate some genetic information (47). This is supported by the observation that the types of tRNA synthetases present in a cell change with age (47). The error theory proposes that aging and subsequent death of a cell are the result of errors that may occur at any step in the sequence of information transfer, resulting in the formation of an altered protein. Inaccurate protein synthesis and inaccurate DNA synthesis are coupled phenomena (48). It becomes difficult to separate their effects, emphasizing the need to include both types of errors in an attempt to explain cellular aging. The cross-linking theory suggests that the accumulation of cross-linked molecules may be responsible for the physical changes that occur during aging (49). Many macromolecules develop stable cross-linkages—either intramolecular or intermolecular—over time. The formation of cross-links alters the physiochemical properties of molecules, thereby affecting their normal function. The nonenzymatic addition of glucose to proteins and DNA serves as an example (50,51).

The initial reaction of glucose with a protein involves the formation of a Schiff base with the ϵ-amino groups of lysine or N-terminal α-amino groups. This complex then rearranges to a more stable Amadori product. Amadori products can be converted to "advanced glycosylation end products" which cross-link proteins (51). In diabetes the rate of age-associated collagen cross-linking is greatly accelerated (52). Cerami and co-workers (50) have shown that aminoguanidine, a nucleophilic hydrazine compound, inhibits advanced glycosylation product formation and glucose-derived cross-linking *in vitro* and *in vivo* when administered to diabetic rats. Their results demonstrate the potential of aminoguanidine, or related compounds, to prevent reduced arterial elasticity, increased peripheral vascular resistance, arteriosclerosis, and capillary basement membrane thickening in diabetes and aging (50).

The aging of some cells has been associated with the accumulation of modified, catalytically inactive forms of several enzymes (41). These altered enzymes generally exhibit a reduced specific activity and a decrease in their heat stability (53). The inactive forms of many enzymes that are readily oxidized by MFO systems accumulate with age; among these are enolase, glucose-6-phosphate dehydrogenase, pyruvate kinase, and superoxide dismutase (41). Since MFO systems are ubiquitous among mammals and because it has been demonstrated that many key metabolic enzymes

are susceptible to oxidative inactivation, the accumulation of inactive enzymes during aging may be due in part to MFO system-catalyzed reactions.

Variability in the specific activity of apparently homogeneous preparations of glutamine synthetase from different batches of *E. coli* is accounted for by the presence of oxidized enzyme with the same characteristics as the inactive form generated *in vitro* by MFO-catalyzed oxidation reactions (37). The difference in specific activity can be quantitatively evaluated by the extent of reaction with carbonyl reagents (37). This could account for the reduced specific activity observed with other enzymes, if they too can be oxidatively inactivated.

It has been suggested that the decrease in heat stability of enzymes, with age, reflects spontaneous time-dependent changes in protein conformation which become more evident when the half-lives of the proteins are increased via a decrease in the rate of protein degradation (53). To determine if similar changes in stability can be caused by MFO-catalyzed oxidation of proteins, glucose-6-phosphate dehydrogenase was incubated with a MFO system comprised of ascorbate, Fe(III), O_2, and EDTA (41). A biphasic pattern of enzymatic heat inactivation similar to that observed for proteins from "old" erythrocytes and cultured fibroblasts from patients with accelerated aging diseases was observed. Unoxidized glucose-6-phosphate dehydrogenase exhibits heat-inactivation kinetics similar to that observed for the enzyme isolated from "young" erythrocytes. Conformational changes leading to the generation of less heat-stable forms of some proteins can be obtained by exposure to MFO systems.

If oxidative modification is a signal for protein degradation under normal conditions, then as aging proceeds it is these otherwise normal intermediates in the degradative pathway that accumulate due to some perturbation. Any one of the following could perturb the pathway: (i) a decrease in the activity and/or amount of specific proteases that attack the marked enzyme; (ii) a decrease in intracellular levels of agents that protect the enzyme from inactivation; (iii) a loss in the ability of catalase, superoxide dismutase, glutathione peroxidase, or nonenzymatic antioxidants to scavenge reactive oxygen species generated by MFO systems; (iv) an increase in the levels and/or activities of mixed-function oxidases that catalyze the reactions, leading to unusually high rates of oxidative damage.

The assertion that the accumulation of inactive enzymes may be due to loss of protection against oxidative damage (iii) is supported by the finding that the amounts of catalase, superoxide dismutase, and glutathione peroxidase decrease with age (54). The importance of nonenzymatic antioxidants has also been demonstrated. Calvin and coworkers (55) have shown that L-buthionine sulfoximine, a specific inhibitor of glutathione biosynthesis, induces severe glutathione depletion and age-specific pathological changes when administered to suckling rats. One of their most striking observations was the development of dense cataracts that paralleled the depletion of lens glutathione. Nutritional imbalances or metabolic disorders can explain the loss of protection brought about by decreases in the concentrations of substrates and cofactors (ii). Further, induction of mixed function oxidases brought about by xenobiotics, toxins, and carcinogens could potentially cause the accumulation of oxidized, inactive enzymes (iv).

The concentration of protein carbonyl groups in cultured human fibroblasts increases progressively with age (41). Cultured fibroblasts from patients with progeria and Werner's syndrome—genetic diseases characterized by accelerated aging—have been used as models for aging studies. Preliminary experiments show that the levels of protein carbonyl groups in tissue samples from patients with progeria or Werner's syndrome are double those in cultured cells from age- and sex-matched normal controls (40). However, evidence for the role of MFO systems in generating the accumulated, oxidized proteins is still inconclusive.

B. Relationship Between the Oxidation of Proteins and Food and Drug Metabolism

If MFO-catalyzed oxidation of proteins is a signal for protein degradation, then protein turnover will be influenced by factors that affect MFO activity (24). Thus, the intake of certain food and drugs could affect protein degradation. The administration of drugs that induce the P-450 system (e.g., barbiturates) could lead to increased protein degradation. The addition of hexobarbital to a reconstituted P-450 system stimulates the oxidative inactivation of GS threefold (40). Hexobarbital is presumed to bind to P-450, leading to a partial uncoupling of substrate oxidation and to a concomitant increased production of hydrogen peroxide (40). Exposure to toxins and carcinogens that induce P-450 activity may also lead to increases in protein degradation, because of the lack of specificity of most P-450 isozymes. High protein-to-carbohydrate ratios increase microsomal MFO activity in humans (54), suggesting that diets high in protein may lead to an increase in the turnover of endogenous proteins. This is a rather simplistic hypothesis, as other regulatory controls have not been explicitly considered (e.g., protection by substrates and the physiological concentrations of modulators of MFO activity). However, it is an easily testable hypothesis. A reconstituted P-450 system (as described in Section III, A) can be supplemented with agents that induce MFO activity, and the oxidative inactivation of GS, or other MFO inactivatible enzymes, then monitored.

C. Neutrophil Action

Oxidative inactivation may play an important role in the protection of higher organisms against bacterial infection. Flavoproteins with NAD(P)H oxidase activity catalyze the formation of activated oxygen species when polymorphonuclear leukocytes (neutrophils) are stimulated to undergo oxidative burst (55). The activated oxygen species generated can diffuse from the neutrophil, enter a target cell, and oxidize proteins with the target cell (56). Incubation of activated neutrophils with intact cells of *E. coli* causes a loss of bacterial GS activity (28). Disruption of bacterial metabolism by the inactivation of GS and other key metabolic enzymes may be partially responsible for the bactericidal action of neutrophils.

It has been shown that phagocytosing neutrophils inactivate their own lysosomal enzymes in an oxygen-dependent reaction (57). Thus, oxidative inactivation may also protect

higher organisms from autolysis by lysosomal enzymes released from their own activated neutrophils.

V. SUMMARY

A number of different MFO systems catalyze the oxidative inactivation of a variety of enzymes. These MFO systems are widely distributed in mammals and many are involved in the metabolism of xenobiotics. Oxidation renders proteins more susceptible to proteolysis. The contention that oxidation serves as a signal for protein degradation is supported by the existence of proteases that preferentially degrade the oxidized form of GS. Many of the enzymes that are readily oxidized by MFO systems accumulate in their inactivated forms during aging. Compounds that induce MFO systems may increase protein degradation. Much work has focused on the metabolism of drugs, but it is also important to consider other processes that may be occurring simultaneously, namely, the degradation of endogenous proteins.

ACKNOWLEDGMENTS

I would like to thank John Cashman, Phyllis Kosen, and Wolfgang Sadee for their valuable suggestions.

REFERENCES

1. W. Sadee. *Pharm. Res.* 3:3–6 (1986).
2. E. Reich. In R. D. Berlin, H. Herrmann, E. H. Lepow, and J. M. Tanzer (eds.), *Molecular Basis of Biological Degradative Processes,* Academic Press, New York, 1978, pp. 155–169.
3. J. Kenny. *TIBS* 11:40–42 (1986).
4. L. E. Orgel. *Proc. Natl. Acad. Sci. USA* 49:517–521 (1963).
5. A. L. Goldberg. *Proc. Natl. Acad. Sci. USA* 69, 422–426 (1972).
6. G. McLendon and M. Smith, *J. Biol. Chem.* 253: 4004–4008 (1978).
7. G. McLendon. *BBRC* 77:959–966 (1977).
8. T. E. Crieghton. *Proteins: Structures and Molecular Properties,* W. H. Freeman, New York, 1983.
9. G. McLendon and E. Radany. *J. Biol. Chem.* 253:6335–6337 (1978).
10. H. Holzer and P. C. Heinrich. *Annu. Rev. Biochem.* 49:63–91 (1980).
11. H. I. Segal, J. R. Winkler, and M. P. Miyagi. *J. Biol. Chem.* 249:6364–6365 (1974).
12. A. Bachmair, D. Finley, and A. Varshavsky. *Science* 234:179–186 (1986).
13. J. G. Dice and A. L. Goldberg. *Proc. Natl. Acad. Sci. USA* 72:3893–3897 (1975).
14. J. F. Dice, P. Dehlinger, and R. Schimke, *J. Biol. Chem.* 248:4220–4228 (1973).
15. S. Rogers, R. Wells, and M. Rechsteiner. *Science* 234:364–368 (1986).
16. T. Toyo-Oka. *BBRC* 107:44–50 (1982).
17. M. K. Offerman, M. J. McKay, M. W. Marsh, and J. S. Bond. *J. Biol. Chem.* 259:8886–8891 (1984).
18. W. Hood, E. De La Morena, and S. Grisolia. *Acta Biol. Med. Germ.* 36:1667–1672 (1977).
19. D. A. Bernlohr and R. L. Switzer. *Biochemistry* 20:5675–5681 (1981).
20. A. Hershko and A. Ciechanover. *Annu. Rev. Biochem.* 51:335–364 (1982).
21. J. W. Payne and J. M. Tuffnell. *FEMS Microbiol. Lett.* 12:279–282 (1981).
22. A. B. Robinson, J. H. McKerrow, and P. Cary. *Proc. Natl. Acad. Sci. USA* 66:753–757 (1970).
23. C. M. Gundberg and P. M. Gallop. *Fed. Proc.* 43:1671 (1984).
24. E. R. Stadtman, *Trends Biochem. Sci.* 11,11–13 (1986).
25. E. R. Stadtman and A. Ginsberg. In P. D. Boyer (ed.), *The Enzymes,* 3rd ed., Academic Press, New York, 1974, Vol. 10, pp. 755–807.
26. R. L. Levine, C. N. Oliver, R. M. Fulks, and E. R. Stadtman. *Proc. Natl. Acad. Sci. USA* 78:2120–2124 (1981).
27. R. L. Levine. *J. Biol. Chem.* 258:11828–11833 (1983).
28. L. Fucci, C. N. Oliver, M. J. Coon, and E. R. Stadtman. *Proc. Natl. Acad. Sci. USA* 80:1521–1525 (1983).
29. B. Halliwell, R. F. Pasternack, and J. D. Rycker. In T. E. King, H. S. Mason, and M. Morrison (eds.), *Oxidases and Related Redox Systems,* Pergamon Press, Oxford, 1982, pp. 733–744.
30. C. Walling. In T. E. King, H. S. Mason, and M. Morrison (eds.), *Oxidases and Related Redox Systems,* Pergamon Press, Oxford, 1982, pp. 86–97.
31. S. P. Wolff, A. Garner, and R. T. Dean. *Trends Biochem. Sci.* 11:27–31 (1986).
32. S. A. Levine and P. M. Kidd. *Antioxidant Adaptation: Its Role in Free Radical Pathology,* Biocurrents Division, San Leandro, 1985.
33. H. H. Wasserman. *Ann. N.Y. Acad. Sci.* 171:108–119 (1970).
34. E. R. Stadtman and M. E. Wittenberger. *Arch. Biochem. Biophys.* 239:379–387 (1985).
35. M. Tomita, I. Masachika, and T. Ukita. *Biochemistry* 8: 5149:5160 (1969).
36. B. Cooper, J. M. Creeth, and A. S. R. Donald. *Biochem. J.* 228:615–626 (1985).
37. R. L. Levine. *J. Biol. Chem.* 258:11823–11827 (1983).
38. S. Shaltiel and A. Patchornik. *J. Am. Chem. Soc.* 85:2799–2806 (1963).
39. L. K. Low and N. Castagnoli, Jr. in M. E. Wolff (ed.), *Burger's Medicinal Chemistry,* Part I, John Wiley & Sons, New York, 1980, pp. 107–227.
40. K. Nakamura, C. Oliver, and E. R. Stadtman. *Arch. Biochem. Biophys.* 240:319–329 (1985).
41. C. N. Oliver, B. Ahn, M. E. Wittenberger, R. C. Levine, and E. R. Stadtman. In R. C. Adelman and E. R. Dekker (eds.), *Modern Aging Research: Modification of Proteins During Aging, Vol. 7,* 1985, pp. 39–52.
42. R. C. Bray, S. H. Cockle, E. M. Fielden, P. B. Roberts, G. Rotilio, and L. Calabrese. *Biochem. J.* 139:43–48 (1974).
43. A. J. Rivett. *J. Biol. Chem.* 260:300–305 (1985).
44. S. Ohkuma and B. Poole. *Proc. Natl. Acad. Sci. USA* 75:3327–3331 (1978).
45. K. Nakamura and E. R. Stadtman. *Proc. Natl. Acad. Sci. USA* 81:2011–2015 (1985).
46. N. W. Shock. In J. R. Florini (ed.), *CRC Handbook of Biochemistry in Aging,* CRC Press, Boca Raton, 1981, pp. 271–282.
47. B. L. Strehler. *Time, Cells and Aging,* 2nd ed., Academic Press, New York, 1977.
48. L. E. Orgel. *Nature* 243:441–445 (1973).
49. J. Bjorksten. *J. Am. Geriat. Soc.* 16:408–427 (1968).
50. M. Brownlee, H. Blassara, A. Kooney, P. Ulrich, and A. Cerami. *Science* 232:1629–1632 (1986).
51. A. Cerami, *Trends Biochem. Sci.* 11:311–314 (1986).
52. J. J. Yunis and O. Prakash. *Science* 215:1525–1527 (1982).
53. M. Rothstein, In J. R. Florini (ed.), *CRC Handbook of Biochemistry in Aging,* CRC Press, Boca Raton, Fla., 1981, pp. 103–111.
54. G. Glass and D. Gershon. *Biochem. J.* 218:531–537 (1984).
55. H. I. Calvin, C. Medvedovsky, and B. V. Worgul. *Science* 233:553–555 (1986).
56. S. D. Nelson. In M. E. Wolff (ed.), *Burger's Medicinal Chemistry,* John Wiley & Sons, New York, 1980, pp. 227–271.
57. J. A. Badwey and M. L. Karnovsky. *Annu. Rev. Biochem.* 49:695–726 (1980).
58. S. J. Weiss. *J. Biol. Chem.* 255:9912–9917 (1980).
59. A. A. Voetman, R. S. Weening, M. M. Hamers, L. J. Meerhof, A. A. A. M. Bot, and D. Roos. *J. Clin. Invest.* 67:1541–1549 (1981).