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Covalent Modification Reactions Are Marking Steps in Protein Turnover

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In pioneering studies Schoenheimer (1946) discovered that intracellular proteins undergo continuous synthesis and degradation. In the meantime, it has been demonstrated that various enzymes turnover at different rates and that the turnover of a particular enzyme may be selectively influenced by nutritional factors, cellular differentiation, organelle distribution, environmental conditions, and metabolic abnormalities of genetic or nongenetic origins. Attempts to identify structural features of proteins that dictate turnover rates have on the whole been disappointing. However, rates of turnover in vivo vary with respect to molecular size, isoelectric point, hydrophobicity, the N-terminus amino acid, thermal stability, conformational changes provoked by protein–protein and protein–lipid interactions, and the concentrations of coenzymes, allosteric effectors, and enzyme substrates. For general reviews, see Schimke and Doyle (1970), Pine (1972), Goldberg and Dice (1974), Schimke and Bradley (1975), Goldberg and St. John (1976), Holzer and Heinrick (1980), and Rivett (1986). Nevertheless, exceptions to all of these generalizations have frustrated attempts to correlate protein turnover with unique structural features. Likewise, attempts to identify the proteolytic systems involved in protein turnover have led to the realization that no single system in responsible. Indeed, it is now evident that ATP-dependent and ATP-independent, lysosomal and nonlysosomal, and ubiquitin-dependent and ubiquitin-independent pathways are all involved in protein turnover at one stage or another.

It is particularly significant that the rates of turnover of various proteins in vivo correlate reasonably well with their susceptibilities to degradation by endopeptidases in vitro (Dice et al., 1973; Segal et al., 1974), and there is a reasonable correlation between the rates of degradation of various enzymes in vivo and their thermal stabilities (McLendon & Radany, 1978); moreover, abnormal and denatured proteins are degraded more rapidly than their native counterparts. Thus, proteins containing amino acid substitutions resulting from site-specific mutations or the incorporation of amino acid analogues (viz., canavalin for arginine, azetitinecarboxylic acid for cysteine, fluorophenylalanine for phenylalanine) are degraded more rapidly than their normal counterparts.

Because the posttranslational modification of a given amino acid residue in a protein is equivalent to the substitution of that residue by an analogue, it has been proposed that the covalent modifications of amino acid residues may serve as "marking" steps for protein degradation (Hood et al., 1977; Holzer & Heinrich, 1980; Oliver et al., 1980; Levine et al., 1981).

This concept is confirmed by the results of in vitro experiments showing that several kinds of covalent modification increase the sensitivity of various enzymes to degradation by purified proteases [for reviews, see Rivett (1986) and Stadtman (1986, 1988a,b)]. Such modifications include the oxidation of amino acid residues by activated oxygen species, the esterification of hydroxyl groups (adenylylation of tyrosine residues, phosphorylations of serine or threonine residues), the acylation (carbamylation, acetylation, ubiquitination) of N^{ϵ}-lysine residues, the oxidation of non-heme iron-sulfur centers, the amino acylation of N-terminal glutamate or aspartate residues, the deamidation of glutamine and asparagine residues, and the glycosylation of amide or hydroxyl groups [for specific examples, see Rivett (1986), Stadtman (1986, 1988a), Holzer and Heinrick (1980), and Gonda et al. (1989)]. Other modifications that are probable marking steps for proteolytic degradation but that have not been directly tested for this activity include the glycation of N^{ϵ}-lysyl groups, proline isomerization, aspartate racemization, the addition of α,β-unsaturated aldehydes to cysteine sulfhydryl groups (thiol ester formation), the reaction of aldehydes with N^{ϵ}-lysyl groups, and various enzymic and nonenzymic reactions leading to inter- and intraprotein cross-linking or to peptide bond cleavage. Because the intracellular accumulation of proteins with these modifications is associated with a loss of endogenous protease activities (viz., with aging), it seems likely that such modifi-

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cations serve as marking steps for protein degradation.

Oxidative Denaturation Marks Enzymes for Proteolysis. Studies on the regulation of enzyme turnover in *Escherichia coli* and *Klebsiella aerogenes* led to the discovery that the intracellular degradation of several enzymes is preceded by oxidative modifications catalyzed by a mixed-function oxidation (MFO) system comprised of NAD(P)H, O_2, Fe(III) or Cu(II), and endogenous enzymes [presumably NAD(P)H oxidases]. Subsequently, it was found that a variety of enzymic and nonenzymic MFO systems, now referred to as metal-ion-catalyzed oxidation (MCO) systems (Amici et al., 1989), have the capacity to catalyze oxidative modifications of proteins (Oliver et al., 1980; Levine et al., 1981; Fucci et al., 1983; Stadtman & Wittenberger, 1985). From these studies, it was concluded that the H_2O_2 and Fe(II) or Cu(I) generated by the MCO systems interact at metal binding sites on proteins to form activated oxygen species ($\dot{O}H$, O_2^-, ferryl ions), which oxidize the side chains of amino acid residues at the metal binding sites. It was proposed that the oxidation is one modification that "marks" an enzyme for proteolytic degradation (Oliver et al., 1980; Levine et al., 1981; Rivett et al., 1985). In the meantime, this concept gained support from studies showing that neutral proteases with high specificity for the oxidized forms of proteins are widely distributed (Rivett et al., 1985; Rivett, 1985a,b; Roseman & Levine, 1987; Davies, 1987; Davies & Goldberg, 1987; Lee et al., 1988; Davies & Lin, 1988; Marcillat et al., 1988) and that the oxidized forms of purified enzymes are degraded more rapidly than their native counterparts by any one of several common proteases, viz., trypsin, pepsin, subtilisin, calpain, and cathepsin D (Farber & Levine, 1982; Rivett, 1985b; Dean & Pollak, 1985; Hunt et al., 1988).

Mechanisms of Oxygen-Radical-Mediated Modifications. Histidine, proline, arginine, lysine, methionine, and cysteine residues are particularly sensitive to site-specific metal-ion-catalyzed oxidation. Levine (1983) showed that some amino acid residues are converted to carbonyl derivatives. It is evident from studies from homopolymers of amino acids that carbonyl derivatives are formed in the metal-ion-catalyzed oxidation of proline, arginine, lysine, and histidine residues (Oliver et al., 1985; Amici et al., 1989). Glutamic semialdehyde residues have been identified as products of proline and arginine oxidation (Amici et al., 1988; Climent et al., 1989), and an adipic semialdehyde residue is tentatively identified as the product of lysine oxidation (Miller, Berlett, and Stadtman, unpublished data). Presumptive evidence that threonine residues may be oxidized to 2-amino-3-ketobutyric acid residues has also been presented (Taborsky, 1973). In addition to carbonyl derivatives, proline residues are converted also to glutamic acid and/or pyroglutamic acid residues, and histidine residues are converted to asparagine and/or aspartic acid residues (Farber & Levine, 1986; Creeth et al., 1983; Cooper et al., 1985).

Methionine residues are oxidized to methionine sulfoxide derivatives (Maier et al., 1989; Auroma & Halliwell, 1989), and cysteine residues are converted to mixed disulfides and to inter- and intra-disulfide cross-linkages (Ballard & Hopgood, 1976; Francis & Ballard, 1980; Bond & Offermann, 1981; Offerman et al., 1984).

It is noteworthy that tryptophan, phenylalanine, and tyrosine residues in proteins are not major sites of oxidation by metal-ion-catalyzed oxidation systems. In contrast, all amino acid residues are modified by radicals produced during γ-radiolysis, but in this case the aromatic amino acids are the preferred targets [for review, see Swallow (1960)]. These differences in target specificity are probably attributable to the site-specific nature of the metal-ion-catalyzed reactions as noted above. In the absence of O_2, $\dot{O}H$ radicals produced by radiolysis lead to extensive protein–protein cross-linkage via tyrosine–tyrosine bonding, and possibly other amino acid cross-links as well (viz., –S–S– cross-links). In the presence of O_2, the cross-linking reactions are suppressed, and considerable peptide bond cleavage occurs, with concomitant formation of protein carbonyl groups (Garrison et al., 1962; Schuessler & Schilling, 1984; Wolff et al., 1986; Davies et al., 1987). On the basis of results from studies with amino acids and amines, it is generally agreed that peptide bond cleavage probably involves $\dot{O}H$-mediated hydrogen abstraction from the α-carbon atom to form carbon-centered radicals; these in the presence of O_2 will form peroxy radical intermediates, which can facilitate peptide bond cleavage to peptide amide and carbonyl peptide derivatives. One of several mechanisms proposed by Garrison et al. (1962) is

$$H_2O + O_2 \rightarrow \dot{O}H + H\dot{O}_2 \quad (1)$$

$$RCONHC(R^2)HCONHR^3 + \dot{O}H \rightarrow RCONH\dot{C}(R^2)CONHR^3 + H_2O \quad (2)$$

$$RCONH\dot{C}(R^2)CONHR^3 + O_2 \rightarrow RCONHC(R^2)(\dot{O}_2)CONHR^3 \quad (3)$$

$$RCONHC(R^2)(\dot{O}_2)CONHR^3 + H\dot{O}_2 \rightarrow RCONHC(R^2)(OOH)CONHR^3 + O_2 \quad (4)$$

$$RCONHC(R^2)(OOH)CONHR^3 \rightarrow RCON{=}C(R^2)CONHR^3 + H_2O_2 \quad (5)$$

$$RCON{=}C(R^2)CONHR^3 + H_2O \rightarrow RCONH_2 + R^2COCONHR^3 \quad (6)$$

The overall reaction is formally analogous to the oxygen-dependent, site-specific metal-ion-catalyzed reaction described by Bateman et al. (1985) and the oxidative cleavage of peptide–copper complexes described by Levitzki et al. (1967).

Subsequently, Schuessler and Schilling (1984) noted that the number of peptide fragments obtained upon radiolysis of bovine serum albumin is approximately equal to the number of prolyl residues in the protein and postulated that a preferred target of radiation-induced chain scission "may be the amino acyl–proline bond, because tertiary amide bonds are easier to oxidize than secondary amide bonds." As noted by Wolff et al. (1986), the possibility that peptide bond cleavage is due to oxidation of the proline moiety itself must also be considered, but see below.

Deamidation of Asparaginyl and Glutaminyl Residues. Asparaginyl and glutaminyl residues undergo spontaneous deamidation at rates determined by the amino acid sequences. Robinson and co-workers [see Robinson and Rudd (1974)] examined 70 different pentapeptides containing either an asparaginyl or a glutaminyl residue in the central position and showed that their rates of deamidation varied from 6 days to 9 years. They proposed that sequences around asparaginyl and glutaminyl residues may have been selected through evolutionary pressures to serve as biological clocks that specify the rates of protein turnover in vivo. There is in fact a positive correlation between the rates of enzyme turnover in vitro and their contents of asparaginyl plus glutaminyl residues (Robinson & Rudd, 1974). Nevertheless, direct evidence that deamidation renders an enzyme more susceptible to proteolytic degradation, though reasonable, is still lacking. A role of deamidation in enzyme turnover is suggested by the fact that the accumulation of deamidated forms of several enzymes varies reciprocally with respect to the intracellular levels of proteases, viz., during aging and in individuals with premature

aging disease [for review, see Gracy et al. (1985a), Dreyfus et al. (1978), and McKerrow (1979)]. The most convincing evidence that deamidation contributes to the pool of altered enzymes comes from studies showing that the age-related accumulation of four more acidic, more labile isoenzyme forms of triosephosphate isomerase in several test systems (Yuan et al., 1981; Gracy et al., 1985a,b) is due to the sequential deamidation of two asparaginyl residues (Asn 71 and Asn 15). The observation that deamidation destabilizes subunit interactions and that the monomeric subunit is more susceptible to proteolytic degradation (Yuan et al., 1981) supports the thesis that deamidation "marks" enzymes for degradation. This thesis is also supported by the fact that the rate of deamidation (8 days) of rabbit muscle aldolase is about the same as the half-life of the enzyme in vivo (Midelfort & Mehler, 1972) and by the studies of Gonda et al. (1989) showing that enzymes in reticulocyte lysates and in yeast catalyze the deamidation of N-terminal asparaginyl and glutaminyl residues of mutant forms of β-galactosidase. The resulting N-terminal Asp and Glu are then susceptible to aminoacylation by Arg-tRNA- or Lys-tRNA-specific transferases to generate protein derivatives with destabilizing N-terminal Arg or Lys residues. Thus modified, the β-galactosidase becomes susceptible to ubiquitin conjugation and subsequent degradation by the ATP-dependent ubiquitin pathway (see below). The generality of the N-terminal Glu or Asp aminoacylation pathway for the degradation of other proteins remains to be determined.

Dean (1987) and Wolff et al. (1986) proposed that N-terminal glutamyl and aspartyl residues generated in the oxidative cleavage of prolyl and histidyl residues, respectively, might be targeted for degradation by the above Arg-modification mechanism. It is noteworthy, however, that neither glutamyl nor aspartyl residues have been shown to be primary products of the metal-ion-catalyzed oxidation reactions, nor have they been identified as N-terminal residues of fragments obtained by oxygen-radical-mediated peptide bond scission. The primary products of oxidation are more likely pyroglutamyl (Amici et al., 1989) and asparaginyl (Farber & Levine, 1986) residues, which are converted to glutamic and aspartic acids during the hydrolysis procedures used in their identification. Whereas polyhistidine has been shown to undergo fragmentation by metal-ion-catalyzed reactions and aspartate has been identified in the acid hydrolysates of the oxidized polymer (Cooper et al., 1985), the oxidation of histidyl to asparaginyl residues in proteins, at least in the case of glutamine synthetase, is not associated with peptide bond cleavage (Farber & Levine, 1986). The N-terminal pyroglutamyl peptide that could be formed in the oxidation of prolyl residues in protein (Amici et al., 1989) might resist degradation by the ubiquitin pathway because of a blocked *N*-amino group (Herschko et al., 1984). Moreover, scission of prolyl peptide bonds by Garrison's mechanism, reactions 1–6, would not lead to an N-terminal glutamate residue.

Racemization and Isomerization of Aspartyl and Asparaginyl Residues. Proteins containing D-aspartyl and/or isoaspartyl residues have been shown to accumulate in eye lens (Masters et al., 1977), in teeth (Helfman & Bada, 1975, 1976), and in human erythrocyte membrane during aging (Brunauer & Clarke, 1986). Geiger and Clark (1987) demonstrated that these derivatives as well as the deamidation of asparaginyl residues likely occur via a common succinimide intermediate, as shown in Figure 1. The carboxyl groups of these abnormal aspartyl derivatives are readily methylated by highly specific *S*-adenosylmethionine-dependent carboxyl methyltransferases. Because L-aspartyl residues are not substrates, methylation of protein in the presence of the transferase and [*methyl*-^{14}C]-*S*-adenosylmethionine provides a highly sensitive measure of abnormal aspartyl residues in protein (Clarke, 1985). Johnson et al. (1987) have shown that cycles of carboxylmethylation can facilitate the conversion of the D-aspartyl and isoaspartyl residues back to the L-aspartyl configuration.

Prolyl Isomerization. Amino acid residues in proteins and peptides exist mainly in the trans configuration. However, depending upon amino acid composition and sequence, the prolyl residues of naturally occurring proteins may be stabilized in the cis configuration [for review, see Stadtman (1988a)]. The potential importance of cis–trans prolyl isomerization in protein degradation is suggested by the fact that chymotrypsin and other proline-specific endopeptidases are able to split prolyl bonds only if the prolyl residue is in the trans configuration (Fischer et al., 1983, 1984; Bachinger, 1987); the trans configuration is essential also to enable a protein to assume a triple-helix configuration. The biological significance of cis–trans isomerization in the proper folding of proteins is highlighted by the discovery of a highly specific prolyl isomerase (Fischer & Bang, 1984; Bachinger, 1987; Lang et al., 1987) and the identification of this isomerase as the cyclosporin receptor protein (Takahashi et al., 1989).

Ubiquitin-Dependent Proteolysis. The conjugation of ubiquitin to the ε-amino group of lysyl residues of some denatured proteins facilitates their degradation (Ciechanover et al., 1980, 1984; Wilkinson et al., 1980; Chin et al., 1982). The ubiquitination is a multistep process in which the C-terminal glycine carboxyl group of ubiquitin (Ub–COOH) is activated by an activating enzyme (E1–SH) to form an enzyme-bound Ub–C(O)–AMP (reaction 7). This is followed by thiolytic

$$\text{E1–SH} + \text{Ub–COOH} + \text{ATP} \rightarrow \text{Ub–C(O)–AMP·E1–SH} + \text{PP}_i \quad (7)$$

$$\text{Ub–C(O)–AMP·E1–SH} \rightarrow \text{E1–S–C(O)–Ub} + \text{AMP} \quad (8)$$

$$\text{E1–S–C(O)–Ub} + \text{E2–SH} \rightarrow \text{E2–S–C(O)–Ub} \rightarrow \text{E1–SH} \quad (9)$$

$$\text{E2–S–C(O)–Ub} + \text{Pr–NH}_2 \xrightarrow{\text{E3}} \text{Pr–NH–C(O)–Ub} + \text{E2–SH} \quad (10)$$

$$\text{Pr–NHC(O)–Ub–NH}_2 + n[\text{E2–S–C(O)–Ub}] \xrightarrow{\text{E3}} \text{Pr–NHC(O)–Ub–NH–[C(O)–Ub]}_n \quad (11)$$

cleavage of the Ub–C(O)–AMP bond by a sulfhydryl group on E1–SH to form a ubiquitin thiolester, E1–S–C(O)–Ub (reaction 8). The ubiquitin moiety of E1–S–C(O)–Ub is then transferred to the sulfhydryl group of a small ubiquitin carrier protein (E2–SH), reaction 9, and then to a particular ε-amino group (Pr–NH_2) of the target protein (reaction 10); this reaction is catalyzed by a specific ubiquitin transferase, E3. Whereas monoubiquitination of proteins is implicated in diverse physiological processes [see Cook and Chock (1988), Rechsteiner (1988), Schlesinger and Herschko (1988), and Ciechanover and Schwartz (1989)], it is insufficient to target denatured proteins for degradation. To be recognized by the ubiquitin–ATP-dependent proteolytic system, several ubiquitin molecules must be conjugated to the target protein. Using genetically engineered constructs of β-galactosidase, Chau et al. (1989) established that only one lysyl residue (residue 15 or 17) of β-galactosidase is modified. Multiply ubiquitinated conjugates are formed by the subsequent addition of up to 20 ubiquitin peptides to the ubiquitin moiety of the monoconjugate (reaction 11). This involves the generation of an ordered

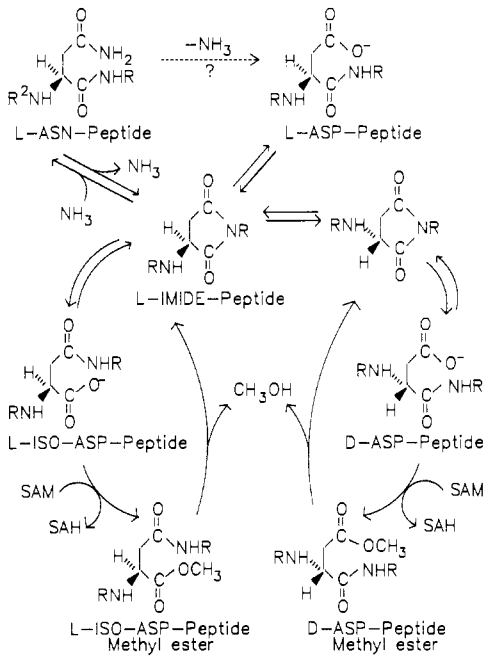


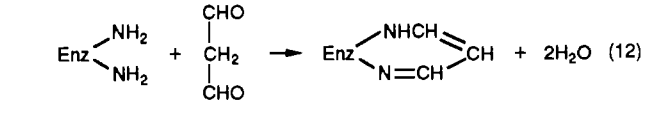
FIGURE 1: Role of succinimide peptide intermediate and carboxylmethylation in the deamidation, racemization, and isomerization of asparaginyl- and aspartyl-containing peptides. Abbreviations: SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

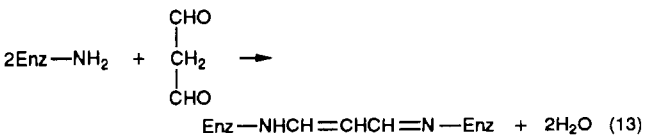
chain of branched ubiquitin–ubiquitin conjugates by a mechanism in which the carboxyl-terminal Gly 76 of one ubiquitin moiety is joined in isopeptide linkage to the internal Lys 48 of an adjacent ubiquitin moiety. In this way, the conjugation of a single Lys residue on the target protein by a highly branched polyubiquitin tree-like structure serves as the secondary "marking" step for proteolytic degradation. The "primary" marking steps, i.e., the denaturing steps that target proteins for ubiquitination, are still poorly defined. In the case of RNase, however, the oxidation of a methionine residue to the sulfoximine derivative renders the enzyme highly susceptible to degradation by the ubiquitin-dependent pathway (Herschko et al., 1986).

Acetylation of the α-amino group of some proteins inhibits their conjugation to ubiquitin and protects them from degradation by the ubiquitin-dependent pathway (Herschko et al., 1984). This focused attention on the importance of an unmodified, exposed amino acid residue at the N-terminus of proteins as a recognition site for the ubiquitin transferase. By means of site-directed mutagenesis, Bachmiar et al. (1986) replaced the N-terminal Met residue of *E. coli* β-galactosidase with other amino acids and found that enzyme species with either Met, Ser, Ala, Thr, Val, Gly, Pro, or Cys at the N-terminus had in vivo half-lives of 20 h or more, whereas the constructs with either Glu, Tyr, Gln, His, Arg, Lys, Phe, Trp, Leu, Asn, or Asp at the N-terminus had very short half-lives (2–30 min). On the basis of these results and correlations with the N-terminal amino acid compositions of the more stable enzymes in vivo, these workers proposed that the half-lives of proteins are specified by the identity of the N-terminal amino acid residue, and they formalized this concept in terms of the so-called "N-end rule". In the meantime, evidence both consistent with and inconsistent with the N-end rule has been obtained [see, for example, Ciechanover and Schwartz (1989), Rogers et al. (1986), Rogers and Rechsteiner (1988), Ciechanover et al. (1984), Bachmair and Varschavsky (1989), Ghoda et al. (1989), Speiser and Etlinger (1983), Saus et al. (1982), and Rote et al. (1989)]. Clearly, structural features of proteins downstream from the N-terminus ("body-type" features) are also important for the recognition of proteins as substrates for ubiquitination and degradation. In fact, body-type proteins appear to be more abundant than N-end-type proteins (Ciechanover & Schwartz, 1989). It is noteworthy, however, that single amino acid substitutions of internal amino acid residues of a protein may alter its thermal stability and susceptibility to proteolytic degradation (Ogasahara et al., 1985; McLendon & Radnay, 1978; Yutani et al., 1977, 1984). It is therefore possible that the effects of N-terminal amino acid substitutions are merely the expression of a more general principle, namely, that the substitution of one amino acid residue in a protein by another amino acid may alter sensitivity of the protein to degradation. A systematic study of the substitution of various amino acids for a particular residue in the body of a protein might disclose correlations similar to those observed for N-end group substitutions [for example, see Yutani et al. (1977)]. If so, the enunciation of a body-rule would be no less justified than the enunciation of an N-end rule.

Schiff Base Formation. The reaction of amino groups of proteins with aldehydes to form Schiff base adducts is well-known. Until now, nonenzymic reactions of malondialdehyde and glucose with proteins are the only ones known to occur in vivo. The possible implication of these reactions in atherogenesis, diabetes, aging, and cataractogenesis has prompted considerable interest in their metabolism.

The potential role of such reactions in targeting proteins for proteolysis is inferred by the fact that macrophages and sinusoidal liver cells possess "scavenger" receptors for the binding, endocytosis, and degradation of these aldehyde-derived adducts. Two kinds of receptors have been identified. One type recognizes malondialdehyde-modified proteins, e.g., the low-density lipoproteins (Fogleman et al., 1980). The other type is implicated in the endocytosis of advanced glycosylation end products (AGE) formed in vivo (Vlassara et al., 1985, 1987), as well as a number of chemically synthesized aliphatic aldehyde–protein conjugates (Horiuchi et al., 1986; Takata et al., 1988). In model studies with ribonuclease A, Chio and Tappel (1969) showed that malondialdehyde formed in the peroxidation of polyunsaturated fatty acids (viz., arachidonic or linolenic acid) reacts with the ϵ-amino groups of lysyl residues in the enzyme (Enz) to form highly fluorescent, catalytically inactive intra- or interprotein conjugated imine adducts (reactions 12 and 13).





Glycation. Glycation of proteins involves the nonenzymic reaction of reducing sugars with the N-terminal α-amino group or, more often, with the ϵ-amino groups of lysyl residues to form Schiff base adducts that undergo rapid Amadori rearrangements to relatively stable ketoamines (Figure 2). These ketoamines are slowly converted to brown, fluorescent polymeric derivatives that are referred to as advanced glycosylation end products (AGE). The overall process is known as the browning reaction or the Maillard reaction. It is responsible for the nonenzymic deterioration of foods during storage and has been implicated in several physiological disorders, viz., diabetes, cataractogenesis, and aging. Moreover, glycated

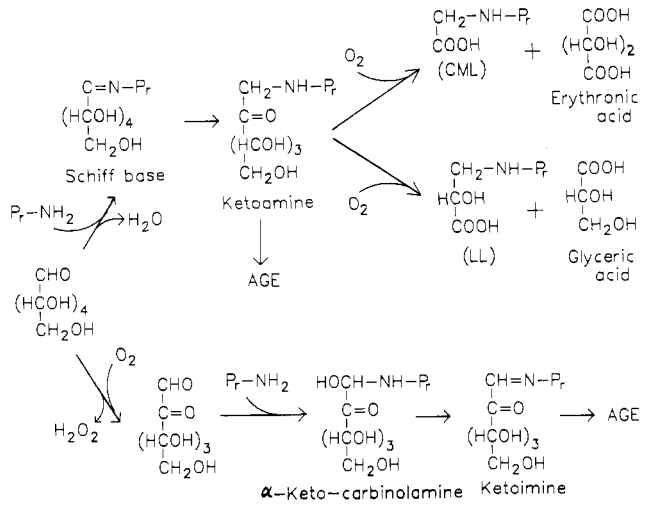


FIGURE 2: Mechanisms of protein glycation. Abbreviations: CML, N^{ϵ}-(carboxymethyl)lysyl residue; LL, 3-(N^{ϵ}-lysino)lactic acid; $PrNH_2$, ϵ-amino group of a protein lysyl residue; AGE, advanced glycosylation end products.

derivatives of several enzymes/proteins have been shown to occur in vivo [for reviews, see Harding (1985), Cerami and Crabbe (1986), and Cerami et al. (1987, 1986)].

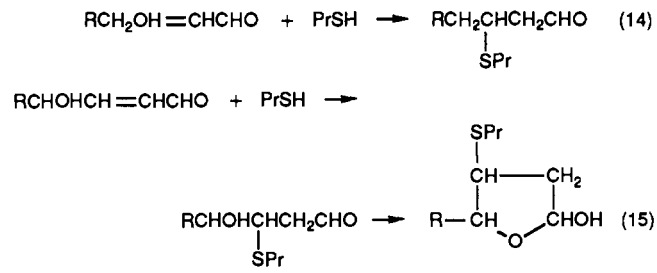
A nonbrowning pathway of glycation was disclosed by the studies of Ahmed et al. (1986, 1988) showing that oxidative cleavage of fructosyllysyl residues in protein is catalyzed by an oxygen-dependent metal ion free radical mechanism, both in vivo and in vitro (Figure 2). Cleavage between C_2 and C_3 of the carbohydrate moiety yields an N^{ϵ}-(carboxymethyl)lysyl (CML) residue and D-erythronic acid, whereas cleavage between C_3 and C_4 yields the 3-(N^{ϵ}-lysino)lactic acid (LL) derivative and D-glyceric acid; both CML and LL were detected in human urine and in glycated protein in vivo.

Wolff and Dean and their co-workers have presented evidence that glucose-derived fluorescent derivatives of proteins as well as fragmentation of the polypeptide chains is mediated by oxygen free radicals generated in the metal-ion-catalyzed oxidation of glucose (Wolff & Dean, 1987; Hunt et al., 1988). They proposed that oxidation of free glucose yields a ketoaldehyde, which subsequently reacts with the protein amino groups to yield an α-ketocarbinolamine derivative. Upon enolization, this would form a ketoimine, which could undergo further reactions to form brown fluorescent products (Figure 2). The associated fragmentation of the protein is presumably due to side reactions involving random attack of the protein by $\dot{O}H$ generated during glucose oxidation. The data presented by Dean and his colleagues are generally consistent with this hypothesis. However, the relative contributions of the oxidative and nonoxidative pathways of protein glycation are under current debate (Harding & Besnick, 1988; Wolff & Dean, 1988).

Because there is a correlation between advanced glycosylation end product (AGE) formation and the protein–protein cross-linking that occurs during aging, during cataract formation, and in tissue degeneration in diabetes, it is believed that glycation facilitates protein cross-linkage. On the basis of their isolation and characterization of 2-furoyl-4(5)-(2-furanyl)-1*H*-imidazole (FFI) from acid hydrolysates of AGE-containing proteins, Cerami et al. (1986, 1987) proposed an attractive mechanism for glycation-dependent cross-linking. However, a role of FFI in protein cross-linking is discounted by results of studies showing that FFI is not a component of glycated proteins but is an artifactual product of the procedures used in its isolation (Horiuchi et al., 1988; Njoroge et al., 1988). A plausible mechanism for the formation of FFI from unlinked glycated proteins has been presented (Njoroge et al., 1988). It is noteworthy that, in addition to glucose, reactions with fructose (Suárez et al., 1989), mannose and frucose (Davis et al., 1989), and glucose 6-phosphate (Beswick & Harding, 1987) have been shown to glycate proteins. Moreover, glycated proteins and adducts formed by the reactions of protein with simple aliphatic aldehydes and ketones (dihydroxyacetone etc.), formaldehyde, glycolaldehyde, and glyceraldehyde all appear to bind at the f-Alb receptor (so-called because it recognizes the formaldehyde-modified albumin) of rat liver sinusoidal cells (Horiuchi et al., 1986). The Schiff base adduct formed in the reactions of glycolaldehyde with protein lysyl residues also undergoes Amadori rearrangement to form an aldoamine, which reacts readily with the lysyl groups of a second molecule of protein to form protein–protein cross-links (Acharya & Manning, 1983).

Other Modifications. There is reason to believe that the protein modifications described in the foregoing sections mark enzymes for degradation. There are a number of other protein modifications that may also render protein susceptible to degradation, but until now their effects on degradation have not been investigated. These include thiolether derivatization of protein sulfhydryl groups, the methylation of carboxyl and amino acid groups, and a variety of enzymic and nonenzymic protein–protein cross-linking reactions.

(*a*) *Thiolether Formation.* Metal-catalyzed oxidation of polyunsaturated lipids leads to numerous products among which are α,β-unsaturated aldehydes and 4-hydroxyalkenals. These unsaturated aldehydes have been shown to react with the sulfhydryl groups of proteins to form thiolether adducts (reactions 14 and 15); the thiolether derivatives produced from



4-hydroxyalkenals may undergo cyclization to form hemiacetals. For reviews, see Esterbauer and Zolner (1989). Because the protein thiolethers contain a reactive aldehydic function, their derivatization with 2,4-dinitrophenylhydrazine has been used as a measure of protein-bound alkenals in tissue extracts (Benedetti et al., 1982). However, protein carbonyl groups are found in the metal-catalyzed oxidation of side chains of amino acid residues, in the oxidative cleavage of proteins by α-amido transfer mechanisms, and in the glycation of proteins (see above). Therefore, more specific assay procedures are needed in order to assess the origins of protein carbonyl groups.

Thiolether adducts of proteins are also formed by the addition of mevalonic acid derived isoprenyl groups to cysteine residues. These are the result of highly specific enzyme-catalyzed posttranslational modifications that have been shown to occur in yeast, Chinese hamster ovary cells, and HeLa cells (Farnsworth et al., 1989; Rilling et al., 1989). They are presumably involved as mating factors but have been implicated also in the modification of the $P21^{ras}$ gene product and in the modification of human lamin B.

(*b*) *Protein–Protein Cross-Linking.* A number of enzymic and nonenzymic posttranslational modifications of proteins lead to intra- and/or interprotein cross-links. As already noted,

the exposure of proteins to oxygen free radicals can lead to intermolecular cross-linkage via tyrosyl–tyrosyl or –S–S bonds. Moreover, malondialdehyde produced in the free radical mediated oxidation of unsaturated fatty acids can facilitate protein–protein cross-links via Schiff base formation (reaction 13). Also, thiolether conjugates obtained by reaction of lipid-derived 4-hydroxyalkenals with protein sulfhydryl groups (reaction 15) can react further with ϵ-amino groups of lysyl residues ($PrNH_2$) of another protein to form Schiff base protein–protein cross-links (reaction 16). Likewise, Schiff

$$RCHOHC(SPr)HCH_2CHO + PrNH_2 \rightarrow RCHOHC(SPr)HCH_2CH{=}NPr + H_2 \quad (16)$$

base cross-links can arise by reaction of the ketoamine adducts generated in the glycation of proteins (Figure 2) with lysyl residues of another protein molecule (reaction 17) or by re-

$$PrNHCH_2C(O)(CHOH)_nCH_2OH + PrNH_2 \rightarrow PrNHCH_2C(NPr)(CHOH)_nCH_2OH + H_2O \quad (17)$$

action of protein carbonyl groups formed by oxygen free radical mediated oxidation of amino acid side chains with the ϵ-amino group of another protein molecule (reaction 18).

$$PrCHO + PrNH_2 \rightarrow PrCH{=}NPr + H_2O \quad (18)$$

Whereas Schiff base cross-links may be relatively labile, subsequent reduction of the Schiff base [viz., by ascorbate; see Tuma et al. (1984)] will yield stable secondary amine-linked conjugates.

In addition to the above nonenzymic protein–protein cross-linking reactions, one should consider also enzyme-catalyzed posttranslational cross-linking reactions, such as those catalyzed by transglutaminase (TG). This enzyme catalyzes substitution of the amide moiety of a protein glutaminyl residue [$PrC(O)NH_2$] with a primary amine (reaction 19) or with the ϵ-amino group of another protein to yield

$$PrC(O)NH_2 + RNH_2 \xrightarrow{TG} PrC(O)NHR + NH_3 \quad (19)$$

$$PrC(O)NH_2 + PrNH_2 \xrightarrow{TG} PrC(O)NHPr + NH_3 \quad (20)$$

$$2PrC(O)NH_2 + NH_2CH_2(CH_2)_nCH_2NH_2 \xrightarrow{TG} PrC(O)NHCH_2(CH_2)_nCH_2NHC(O)Pr + 2\ NH_3 \quad (21)$$

protein–protein conjugates in which the glutaminyl residue of one protein is linked to the lysyl residue of another protein (reaction 20). Moreover, when a simple primary amine is replaced by a polyamine (e.g., putrescine on spermidine), then protein cross-linking can occur by the polyamine bridge (reaction 21). The reactions catalyzed by transglutaminase presumably serve important physiological functions [for reviews, see Placentini et al. (1988), Fesus and Thomazy (1988), Birckbichler et al. (1988), and Lorand (1988)]; however, functionally incompetent protein complexes produced as a consequence of enzymic nonspecificity would be likely candidates for proteolytic degradation. Indeed, cross-linked protein aggregates produced by one ore more of the above cross-linking reactions accumulate during aging and in various disease states, i.e., under conditions where the levels of proteases are known to decline (Starke-Reed & Oliver, 1989; Taylor & Davies, 1989; Gracy et al., 1985a).

General Considerations. (*a*) *Regulatory Role of Covalent Modification Dependent Proteolysis.* As noted by Schimke (1970), the intracellular concentration of an enzyme is determined by the balance between its rates of synthesis and degradation. Whereas various mechanisms for the repression and depression of enzyme synthesis at both transcriptional and translational levels are well recognized, the mechanisms by which cells regulate the rates of degradation of one enzyme with respect to another are not well understood. That such regulation exists is evident from the fact that there are large differences in the rates of turnover of various enzymes and that the rate of degradation of one enzyme can vary independently of another by variations in the nutritional state of the organism. Because the covalent modification of an enzyme can target it for degradation, it is tacitly accepted that the rate of degradation and hence the intracellular level of an enzyme are regulated by covalent modification reactions. The fact that phosphorylation of proteins constitutes a major mechanism for the regulation of diverse biological functions leads to the proposition that phosphorylation might serve to regulate also the degradation of proteins. This suggestion is supported by the demonstration that the phosphorylated forms of some enzymes are more susceptible to proteolysis than their nonphosphorylated counterparts [for review, see Rivett (1986) and Holzer and Heinrich (1980)]; however, earlier evidence that protein phosphorylation is a critical step in the glucose-induced "proteolytic catabolite inactivation" of some enzymes in yeast is being reassessed in light of more recent findings (Holzer, 1989).

The possibility that metal-ion-catalyzed oxidative modification of enzymes can be used for the selective regulation of enzyme degradation is suggested by the demonstration that the substrates of enzymes can protect them from oxidative modification (Levine et al., 1981; Fucci et al., 1983; Stadtman & Wittenberger, 1985). Such metabolite effects could account for the differential responses of various enzyme levels to nutritional deficiencies. For example, starvation for nitrogen results in a decrease in the intracellular level of glutamate, one of the substrates that protects glutamine synthetase against oxidative damage and subsequent degradation. Teleologically, the regulation of an enzyme's susceptibility to degradation by the concentrations of its substrates makes good sense. In the absence of its substrate, an enzyme is biologically inactive, therefore, its selective degradation can have little effect on its biological functions, but by degradation it can yield amino acids and other products needed for the synthesis of other proteins that are essential for survival during this period of stress.

The metal-catalyzed oxidation of enzymes is definitely the basis of a regulatory mechanism in *Klebsiella aerogenes*. This organism uses two different metabolic pathways for growth on glycerol. Under anaerobic conditions, glycerol is metabolized via the pathway glycerol → dehydroxyacetone → dihydroxyacetone-P. When shifted for anaerobic to aerobic condition, the glycerol dehydrogenase that catalyzes the first step in this sequence is inactivated by a hydrogen peroxide dependent mechanism and is subsequently rapidly degraded (Chevalier et al., 1990). Concomitantly, a new set of enzymes is induced that catalyzes the reaction sequence glycerol → glycerol-3-P → dihydroxyacetone-P. In miroorganisms, the switch from anaerobic to aerobic environment often leads to repression of anaerobic genes, the degradation of the anaerobic gene products, and the derepression of a set of genes coding for enzymes needed for aerobic metabolism. The mechanism elucidated in the studies with *K. aerogenes* may therefore be a prototype for the regulation of shifts from anaerobic to aerobic metabolism.

(*b*) *Housekeeping Function.* A substantial fraction of the covalent modification of protein is the result of uncontrolled, spontaneous changes in protein structure or is due to chemical insults (viz., oxygen radical damage) they defy biological defenses. The selective degradation of these nonfunctional modified proteins is therefore the responsibility of intracellular

proteases that can distinguish between "good" and "bad" proteins. The importance of this housekeeping function is evident from the results of studies showing that the intracellular accumulation of catalytically inactive or less active forms of several enzymes which occurs during aging is correlated with an age-dependent decrease in the intracellular levels of the neutral-alkaline proteinase (Starke-Reed & Oliver, 1989). As judged by the carbonyl content of the total cellular protein, it is evident that a considerable portion of the modified proteins that accumulate with age is due to metal-ion-catalyzed reactions. With the assumption that there is on the average one carbonyl group per 50-kDa polypeptide chain, it can be calculated that 40–50% of the cellular protein in old animals is oxidized. This agrees with the finding that the specific activity of several enzymes in cells from old animals is only about half that measured in cells from young animals. Obviously, still unanswered questions are as follows: (a) How much damaged protein (cellular garbage) can a cell tolerate? (b) Is the age-dependent intracellular accumulation of modified protein due to an increase in the rate of covalent modifications or to a decrease in the capacity to degrade the damaged protein? (c) Is the age-dependent decrease in neutral protease activity due to a deficiency in the rate of protease synthesis or to posttranslational modifications (viz., oxidation) of the proteases themselves?

Finally, it is evident that there is a need to develop sensitive techniques for the quantitation ov various kinds of covalently modified proteins in order to evaluate the contributions of each to the overall pool of damaged protein.

(*c*) *How Are Modified Proteins Recognized as Targets for Degradation*? In view of the extraordinary diversity of posttranslational modifications that target proteins for degradation and the fact that the modifications involve alteration of only one or a few amino acid residues in a given protein, it is surprising that so many different proteinases can distinguish between the modified and native forms of proteins. The suggestion that all modified proteins have in common a structural feature that serves as a recognition signal for proteolytic attack is not easily reconciled with the marked differences in peptide bond specificites of the many proteinases that preferentially degrade modified proteins. Perhaps even subtle perturbations of protein structure, as occurs with most posttranslational modification reactions, can lead to a multiplicity of recognition parameters and thus specify interactions with more than one type of proteinase. This appears to be the case with *E. coli* glutamine synthetase. The native form of this enzyme is almost completely resistant to attack by highly purified preparations of the multicatalytic proteinase from rat liver (Rivett, 1985a) and a neutral proteinase from *E. coli* (Roseman & Levine, 1987); however, following its modification by the ascorbate/Fe(III)/O_2 MCO system, the enzyme is readily degraded by both proteinases and is more susceptible to degradation by several secretory proteinases (Farber & Levine, 1982; Rivett, 1985b). Studies of the time course of the changes that occur during exposure to the MFO system (Rivett & Levine, 1990) revealed that the susceptibility to degradation by the multicatalytic proteinase is associated with the loss of two histidine residues per subunit and is independent of changes in the hydrophobicity of the enzyme. In contrast, the susceptibility to degradation by the *E. coli* proteinase is correlated with the conversion of the enzyme to a more hydrophobic form (Cervera & Levine, 1987; Levine, 1989).

References

Acharya, A. S., & Manning, J. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3590–3594.

Ahmed, M. U., Thorpe, S. R., & Baynes, J. W. (1986) *J. Biol. Chem. 261*, 4889–4894.

Ahmed, M. U., Dunn, J. A., Walla, M. D., Thorpe, S. R., & Baynes, J. W. (1988) *J. Biol. Chem. 263*, 8816–8821.

Amici, A., Levine, R. L., Tsai, L., & Stadtman, E. R. (1989) *J. Biol. Chem. 264*, 3341–3346.

Aruoma, O. I., & Halliwell, B. (1989) *FEBS Lett. 244*, 76–80.

Bachinger, H. P. (1987) *J. Biol. Chem. 262*, 17144–17148.

Bachmair, A., & Varshavsky, A. (1989) *Cell 56*, 1019–1032.

Bachmair, A., Finley, D., & Varshavsky, A. (1986) *Science 234*, 179–186.

Ballard, F. J., & Hopgood, M. F. (1976) *Biochem. J. 154*, 717–724.

Bateman, R. C., Jr., Youngblood, W. W., Busby, W. J., Jr., & Kiser, J. S. (1985) *J. Biol. Chem. 260*, 9088–9091.

Benedetti, A., Esterbauer, H., Ferrali, M., Fulceri, R., & Comporti, M. (1982) *Biochim. Biophys. Acta 711*, 345–356.

Beswick, H., & Harding, J. J. (1987) *Biochem. J. 246*, 761–769.

Birckbichler, P. J., Anderson, L. E., & Dell'orco, R. T. (1988) in *Advances in Post-Translational Modifications of Proteins and Aging* (Zappia, V., Galletti, P., Porta, R., & Wold, F., Eds.) pp 109–117, Plenum Press, New York.

Bond, J. S., & Offermann, M. K. (1981) *Acta Biol. Med. Ger. 40*, 1365–1374.

Brunauer, L. S., & Clarke, S. (1986) *J. Biol. Chem. 261*, 12538–12543.

Cerami, A., & Crabbe, M. J. C. (1986) *Trends Pharmacol. Sci. 7*, 271–274.

Cerami, A., Vlassara, H., & Brownlee, M. (1986) *J. Cell. Biochem. 30*, 111–120.

Cerami, A. C., Vlassara, H., & Brownlee, M. (1987) *Sci. Am. 256*, 90–96.

Cervera, J., & Levine, R. L. (1987) *FASEB J. 2*, 2591–2595.

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., & Varshavsky, A. (1989) *Science 243*, 1576–1583.

Chevalier, M., Lin, E. C. C., & Levine, R. L. (1990) *J. Biol. Chem. 265*, 40–46.

Chin, D. T., Kuehl, L., & Rechsteiner, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5857–5861.

Chio, K. S., & Tappel, A. L. (1969) *Biochemistry 8*, 2827–2833.

Ciechanover, A., & Schwartz, A. L. (1989) *Trends Biochem. Sci. 14*, 483–488.

Ciechanover, A., Heller, H., Elias, S., Hass, A. L., & Hershko, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1365–1368.

Ciechanover, A., Finley, D., & Varshavsky, A. (1984) *Cell 37*, 57–66.

Clarke, S. (1985) *Annu. Rev. Biochem. 54*, 479–506.

Climent, I., Tsai, L., & Levine, R. L. (1989) *Anal. Biochem. 182*, 226–232.

Cook, J., & Chock, P. B. (1988) *BioFactors 1*, 133–146.

Cooper, B., Creeth, M., & Donald, A. S. R. (1985) *Biochem. J. 228*, 615–626.

Creeth, J. M., Cooper, B., Donald, A. S. R., & Clamp, J. R. (1983) *Biochem. J. 211*, 323–332.

Davies, K. J. A. (1987) *J. Biol. Chem. 262*, 9895–9901.

Davies, K. J. A., & Goldberg, A. L. (1987) *J. Biol. Chem. 262*, 8227–8234.

Davies, K. J. A., & Lin, S. W. (1988) *Free Radical Biol. Med. 5*, 215–223.

Davies, K. J. A., Delsignore, M. E., & Lin, S. W. (1987) *J. Biol. Chem. 282*, 9902–9907.
Davis, L. J., Hakim, G., & Rossi, C. A. (1989) *Biochem. Biophys. Res. Commun. 160*, 362–366.
Dean, R. T. (1987) *FEBS Lett. 220*, 278–282.
Dean, R. T., & Pollak, J. K. (1985) *Biochem. Biophys. Res. Commun. 126*, 1082–1089.
Dice, J. F., Dehlinger, P. J., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 4220–4228.
Dreyfus, J. C., Kahn, A., & Schapira, F. (1978) *Curr. Top. Cell. Regul. 14*, 143–297.
Esterbauer, H., & Zollner, H. (1989) *Free Radical Biol. Med. 7*, 197–203.
Farber, J. M., & Levine, R. L. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 865.
Farber, J. M., & Levine, R. L. (1986) *J. Biol. Chem. 261*, 4574–4578.
Farnsworth, C. C., Wolda, S. L., Gelb, M. H., & Glomset, J. A. (1989) *J. Biol. Chem. 264*, 20422–20429.
Fesus, L., & Thomazy, V. (1988) in *Advances in Post-Translational Modifications of Proteins and Aging* (Zappia, V., Galletti, P., Porta, R., & Wold, F., Eds.) pp 119–134, Plenum Press, New York.
Fischer, G., & Bang, H. (1984) *Biomed. Biochim. Acta 43*, 1101–1111.
Fischer, G., Heins, J., & Barth, A. (1983) *Biochim. Biophys. Acta 742*, 452–462.
Fischer, G., Bang, H., Berger, E., & Schellenberger, A. (1984) *Biochim. Biophys. Acta 791*, 87–97.
Fogelman, A. M., Shechter, I. S., Hokom, M., Child, J. S., & Edwards, P. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2214–2218.
Francis, G. L., & Ballard, F. J. (1980) *Biochem. J. 186*, 581–590.
Fucci, L., Oliver, C. N., Coon, M. J., & Stadtman, E. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1521–1525.
Garrison, W. M., Jayko, M. E., & Bennett, W. (1962) *Radiat. Res. 16*, 483–502.
Geiger, T., & Clarke, S. (1987) *J. Biol. Chem. 262*, 785–794.
Ghoda, L., Van Daalen Wetters, T., Macrae, M., Ascherman, D., & Coffino, P. (1989) *Science 242*, 1493–1495.
Goldberg, A. L., & Dice, J. F. (1974) *Annu. Rev. Biochem. 48*, 835–869.
Goldberg, A. L., & St. John, A. C. (1976) *Annu. Rev. Biochem. 45*, 747–803.
Gonda, D. K., Bachmair, A., Wünning, I., Tobias, J. W., Lane, W. S., & Varshavsky, A. (1989) *J. Biol. Chem. 264*, 16700–16712.
Gracy, R. W., Yüksel, K. Ü., Chapman, M. L., Cini, J. K., Jahani, M., Lu, H. S., Gray, B., & Talent, J. M. (1985a) in *Modifications of Proteins during Aging*, pp 1–18, Alan R. Liss, New York.
Gracy, R. W., Chapman, M. L., Cini, J. E., Jahani, M., Tollefsbol, T. O., & Yüksel, K. Ü. (1985b) in *Molecular Biology of Aging* (Woodhead, A. D., Blackett, A. D., & Hollaender, A., Eds.) pp 427–442, Plenum Press, New York.
Harding, J. J. (1985) *Adv. Protein Chem. 37*, 247–334.
Harding, J. J., & Beswick, H. T. (1988) *Biochem. J. 249*, 617–618.
Helfman, P. M., & Bada, J. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2891–2894.
Helfman, P. M., & Bada, J. L. (1976) *Nature (London) 262*, 279–281.
Hershko, A., Heller, H., Eytan, E., Kaklij, G., & Rose, I. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7201–7205.
Hershko, A., Heller, H., Eytan, E., & Reiss, Y. (1986) *J. Biol. Chem. 261*, 11992–11999.
Holzer, H. (1989) *Cell. Biol. Rev. 21*, 306–319.
Holzer, H., & Heinrich, P. C. (1980) *Annu. Rev. Biochem. 49*, 63–91.
Hood, W., DeLa Morena, E., & Grisolia, S. (1977) *Acta Biol. Med. Ger. 36*, 1667–1672.
Horiuchi, S., Murakami, M., Takata, K., & Morino, Y. (1986) *J. Biol. Chem. 261*, 4962–4966.
Horiuchi, S., Shiga, M., Araki, N., Takata, K., Saitoh, M., & Morino, Y. (1988) *J. Biol. Chem. 263*, 18821–18826.
Hunt, J. W., Simpson, J. A., & Dean, R. T. (1988) *Biochem. J. 250*, 87–93.
Johnson, B. A., Murray, E. D., Clarke, S., Glass, D. B., & Aswad, D. W. (1987) *J. Biol. Chem. 262*, 5622–5629.
Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature (London) 329*, 268–270.
Lee, Y. S., Park, S. C., Goldberg, A. L., & Chung, C. H. (1988) *J. Biol. Chem. 263*, 6643–6646.
Levine, R. L. (1983) *J. Biol. Chem. 258*, 11823–11827.
Levine, R. L. (1989) *Cell Biol. Rev. 21*, 347–360.
Levine, R. L., Oliver, C. N., Fulks, R. M., & Stadtman, E. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2120–2124.
Levitzky, A., Anbar, M., & Berger, A. (1967) *Biochemistry 6*, 3757–3765.
Lorand, L. (1988) in *Advances in Post-Translational Modifications of Proteins and Aging* (Zappia, V., Galletti, P., Porta, R., & Wold, F., Eds.) pp 79–94, Plenum Press, New York.
Maier, K. L., Matejkova, E., Hinze, H., Leuschel, L., Weber, H., & Beck-Speier, I. (1989) *FEBS Lett. 250*, 221–226.
Marcillat, O., Zhang, Y., Lin, S. W., & Davies, K. J. A. (1988) *Biochem. J. 254*, 677–683.
Masters, P. M., Bada, J. L., & Zigler, J. S., Jr. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1204–1208.
McKerrow, J. H. (1979) *Mech. Ageing Dev. 10*, 371–377.
McLendon, G., & Radany, E. (1978) *J. Biol. Chem. 253*, 6335–6337.
Midelfort, C. F., & Mehler, A. H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1816–1819.
Njoroge, F. G., Fernandes, A. A., & Monnier, V. M. (1988) *J. Biol. Chem. 263*, 10646–10652.
Offerman, M. K., McDay, M. J., Marsh, M. W., & Bond, J. S. (1984) *J. Biol. Chem. 259*, 8886–8891.
Ogasahara, K., Tsunasawa, S., Soda, Y., Yutani, K., & Sugino, Y. (1985) *Eur. J. Biochem. 150*, 17–21.
Oliver, C. N., Levine, R. L., & Stadtman, E. R. (1980) in *Metabolic Interconversion of Enzymes* (Holzer, H., Ed.) pp 259–268, Springer-Verlag, Berlin.
Oliver, C. N., Levine, R. L., & Stadtman, E. R. (1982) in *Experiences in Biochemical Perception* (Ornston, L. N., & Sligar, S. G., Eds.) pp 233–249, Academic Press, New York.
Oliver, C. N., Ahn, B., Wittenberger, M. E., & Stadtman, E. R. (1985) in *Cellular Regulation and Malignant Growth* (Ebasi, S., Ed.) pp 320–331, Springer-Verlag, Berlin.
Oliver, C. N., Levine, R. L., & Stadtman, E. R. (1987) *J. Am. Geriat. Soc. 35*, 947–956.
Pine, M. J. (1972) *Annu. Rev. Microbiol. 26*, 103–126.
Placentini, M., Cerû-Argento, M. P., Farrace, M. G., & Autuori, F. (1988) in *Advances in Post-Translational Modifications of Proteins and Aging* (Zappia, V., Galletti, P., Porta, R., & Wold, F., Eds.) pp 185–196, Plenum Press, New York.

Rechsteiner, M. (1988) *Ubiquitin*, Plenum Press, New York.
Rilling, H. C., Bruenger, E., Epstein, W. W., & Kandutsch, A. A. (1989) *Biochem. Biophys. Res. Commun. 163*, 143–148.
Rivett, A. J. (1985a) *J. Biol. Chem. 260*, 12600–12606.
Rivett, A. J. (1985b) *Arch. Biochem. Biophys. 243*, 624–632.
Rivett, A. J. (1986) *Curr. Top. Cell. Regul. 28*, 291–337.
Rivett, A. J., & Levine, R. L. (1990) *Arch. Biochem. Biophys.* (in press).
Rivett, A. J., Roseman, J. A., Oliver, C. N., Levine, R. L., & Stadtman, E. R. (1985) in *Intracellular Protein Catabolism* (Khairallah, E. A., Bond, J. S., & Bird, J. W. C., Eds.) pp 317–328, Alan R. Liss, New York.
Robinson, A. B., & Rudd, C. J. (1974) *Curr. Top. Cell. Regul. 8*, 247–294.
Rogers, S., Wells, R., & Rechsteiner, M. (1986) *Science 234*, 179–186.
Rogers, S. W., & Rechsteiner, M. (1988) *J. Biol. Chem. 263*, 19850–19862.
Roseman, J. E., & Levine, R. L. (1987) *J. Biol. Chem. 262*, 2101–2110.
Rote, K., Rogers, S., Pratt, G., & Rechsteiner, M. (1989) *J. Biol. Chem. 264*, 9772–9779.
Saus, J., Timoneda, J., Hernando-Yago, J., & Grisolia, S. (1982) *FEBS Lett. 143*, 225–227.
Schimke, R. T., & Doyle, D. (1970) *Annu. Rev. Biochem. 39*, 929–976.
Schimke, R. T., & Bradley, M. O. (1975) in *Proteases and Biological Control* (Reich, E., Rifkin, D. B., & Shaw, E., Eds.) pp 515–530, Cold Spring Harbor Laboratory, New York.
Schlesinger, M. J., & Hershko, A. (1988) *The Ubiquitin System*, Cold Spring Harbor Laboratory, New York.
Schuessler, H., & Schilling, K. (1984) *Int. J. Radiat. Biol. 45*, 267–281.
Segal, H. L., Winkler, J. R., & Miyagi, M. P. (1974) *J. Biol. Chem. 249*, 6364–6365.
Speiser, S., & Etlinger, J. D. (1982) *J. Biol. Chem. 257*, 14122–14127.
Stadtman, E. R. (1986) *Trends Biochem. Sci. 11*, 11–12.
Stadtman, E. R. (1988a) *J. Gerontol. Biol. Sci. 43*, B112–B120.
Stadtman, E. R. (1988b) *Exp. Gerontol. 23*, 237–347.
Stadtman, E. R., & Wittenberger, M. E. (1985) *Arch. Biochem. Biophys. 239*, 379–387.
Starke-Reed, P., & Oliver, C. N. (1989) *Arch. Biochem. Biophys. 275*, 559–567.
Suárez, G., Rajaram, R., Oronsky, A. L., & Gawinowicz, M. A. (1989) *J. Biol. Chem. 264*, 3674–3679.
Swallow, A. J. (1960) in *Radiation Chemistry of Organic Compounds* (Swallow, A. J., Ed.) pp 211–224, Pergamon Press, New York.
Taborsky, G. (1973) *Biochemistry 12*, 1341–1348.
Takahashi, N., Hayano, T., & Suzuki, M. (1989) *Nature (London) 337*, 473–475.
Takata, K., Horiuchi, S., Araki, N., Shiga, M., Saitoh, M., & Morino, Y. (1988) *J. Biol. Chem. 263*, 14819–14825.
Taylor, A., & Davies, K. J. A. (1987) *Free Radical Biol. Med. 3*, 371–377.
Tuma, D. J., Donohue, T. M., Jr., Medina, V. A., & Sorrell, M. F. (1984) *Arch. Biochem. Biophys. 234*, 377–381.
Vlassara, H., Brownlee, M., & Cerami, A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5588–5592.
Vlassara, H., Valinsky, J., Brownlee, M., Cerami, C., Nishimoto, S., & Cerami, A. (1987) *J. Exp. Med. 166*, 539–549.
Wilkinson, K. D., Urban, M. K., & Haas, A. L. (1980) *J. Biol. Chem. 255*, 7529–7532.
Wolff, S. P., & Dean, R. T. (1987) *Biochem. J. 245*, 243–250.
Wolff, S. P., & Dean, R. T. (1988) *Biochem. J. 249*, 618–619.
Wolff, S. P., Garner, A., & Dean, R. T. (1986) *Trends Biochem. Sci. 1*, 28–31.
Yuan, P. M., Talent, J. M., & Gracy, R. W. (1981) *Mech. Ageing Dev. 17*, 151–162.
Yutani, K., Ogasahara, K., Sugino, Y., & Matsushiro, A. (1977) *Nature (London) 267*, 274–275.
Yutani, K., Ogasahara, K., Aoki, K., Kakuno, T., & Sugino, Y. (1984) *J. Biol. Chem. 259*, 14076–14081.