REVIEW

Degradative Covalent Reactions Important to Protein Stability

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Abstract

Commonly observed chemical modifications that occur in proteins during their in vitro purification, storage, and handling are discussed. Covalent modifications described include deamidation and isoaspartate formation, cleavage of peptide bonds at aspartic acid residues, cystine destruction and thiol-disulfide interchange, oxidation of cysteine and methionine residues, and the glycation and carbamylation of amino groups.

Index Entries: Degradative reactions; deamidation; isoaspartate formation; thiol–disulfide interchange; oxidation of cysteine; oxidation of methionine; glycation; carbamylation; protein stability.

1. Introduction

The covalent modification of proteins in vivo has been proposed as a natural mechanism to designate enzymes for turnover *(1)*. Both enzymatic and nonenzymatic pathways of posttranslational modification of proteins have been identified. Spontaneous, nonenzymatic reactions include the deamidation of asparaginyl residues, racemization of aspartyl residues, isomerization of prolyl residues, and glycation of amino groups, as well as site-specific metal catalyzed oxidations (interaction of H_2O_2 and Fe(II) at metal binding sites on proteins) *(1)*. Enzymes have been identified in vivo that specifically interact with covalently modified proteins, including carboxymethyl transferase (which methylates isoaspartyl [iso-Asp] residues) and alkaline protease (which degrades oxidized proteins). It has been proposed that covalent changes caused by in vivo protein oxidation are primarily responsible for the accumulation of catalytically compromised and structurally altered enzymes during aging *(2)*. In addition, protein oxidation may play a role in several pathological states, including inflammatory disease, atherosclerosis, neurological disorders, and cataractogenesis *(3)*.

This article emphasizes the general characteristics of commonly observed chemical modifications in proteins occurring during their in vitro purification, storage, and handling. Although some of these in vitro degradative covalent reactions are similar or identical to ones observed in biological systems, the identity of the most labile amino acids within a protein and their relative reaction rates often can be significantly different. These covalent modifications are potential problems during any protein unfolding and refolding experiment, since the examination of protein stability is often carried out under rather nonphysiological conditions (elevated temperatures, acidic and alkaline pH, the presence of denaturants, exposure to light, and so on).

Advances in our understanding of the weak chemical links of a protein molecule in vitro originate primarily from three areas in which scientists and engineers are attempting to utilize biomolecules for technological purposes:

1. Food scientists are examining changes in food proteins during processing and heating, especially at extremes of temperature and pH *(4)*.
2. Chemists are increasingly exploring the use of enzymes as specific catalysts for organic syn-

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thesis *(5)*, while chemical engineers are utilizing enzymes as biocatalysts to manufacture a variety of chemicals, sweeteners, and detergents *(6,7)*. These studies examine mechanisms of protein inactivation at elevated temperatures and in nonaqueous solvent systems.

3. Recently, the biotechnology and pharmaceutical industries are actively developing naturally occurring peptides and proteins as therapeutic agents. The purification and formulation of these protein drugs requires an understanding of the causes and mechanisms of inactivation in order to develop rational strategies for their stabilization *(8–11)*.

In each of these applications, protein molecules are exposed to nonphysiological conditions resulting in stresses on their structural and chemical integrity that may lead to both their covalent and noncovalent alteration.

The relationship between the conformational stability and chemical integrity of a protein is of particular importance to the understanding of the mechanisms of protein inactivation. On exposure to changes in environmental conditions (elevated temperature, acidic/basic conditions, or the presence of structure perturbing solutes), protein molecules may undergo conformational changes (local changes in secondary and tertiary structure), reversible unfolding (cooperative loss of higher ordered structure), or inactivation (irreversible changes in the structural or chemical integrity of the molecule). Perturbation of protein structure often leads to the exposure of previously buried amino acid residues, facilitating their chemical reactivity. In fact, partial unfolding of a protein is often observed prior to the onset of irreversible chemical or conformational processes *(8,9)*. Moreover, protein conformation generally may control the rate and extent of deleterious chemical reactions. Conversely, chemical changes to the polypeptide backbone or amino acid side chains of a protein may lead to loss of conformational stability. For example, the reduction of disulfides or the oxidation of cysteine residues can induce protein unfolding and aggregation. During the upcoming discussion of the chemical lability of proteins, the interplay between these reactions and protein conformation is emphasized. Obviously, this coupled interaction between these two phenomena has the potential to complicate studies of protein folding and unfolding significantly.

This article is not intended to be a comprehensive survey of the literature. Rather, the orientation of this discussion is toward the types of problems that can be encountered during the typical handling of protein solutions by the laboratory scientist, with particular emphasis on the conditions used to purify proteins and study their stability and protein folding pathways. Illustrative examples from the literature have been selected to emphasize the strategies used to identify chemical degradation in proteins and minimize its occurrence.

2. Deamidation and Isoaspartate Formation

The spontaneous, nonenzymatic deamidation of asparagine residues is one of the most commonly encountered chemical modifications of proteins. Numerous papers and reviews recently have appeared describing examples of deamidation events during either the isolation or storage of proteins *(12–14)*. Deamidation can occur under acidic, neutral, or alkaline conditions, although the chemical mechanism of hydrolysis is strongly dependent on pH *(see below)*. The biological purpose of deamidation in vivo may involve the regulation of protein degradation and clearance, thus serving as a type of "biological clock" *(15)*. In fact, naturally occurring protein methyl transferases have been identified that specifically modify deamidated byproducts, perhaps tagging damaged protein for either repair or clearance *(16)*.

By examining the rate of amide loss for a large series of synthetic pentapeptides of sequence (Gly-X-Asn-X-Gly and Gly-X-Gln-X-Gly) under physiological conditions, Robinson and coworkers *(14,15)* clearly demonstrated the enhanced lability of peptide amides compared to simple aliphatic amides. The asparagine-containing peptides were observed to deamidate about 5- to 10-fold faster than their glutamine counterparts (mean half-life of deamidation of 70 vs 410 d). The deamidation rates for both the aspartyl and glutamyl containing pentapeptides were also

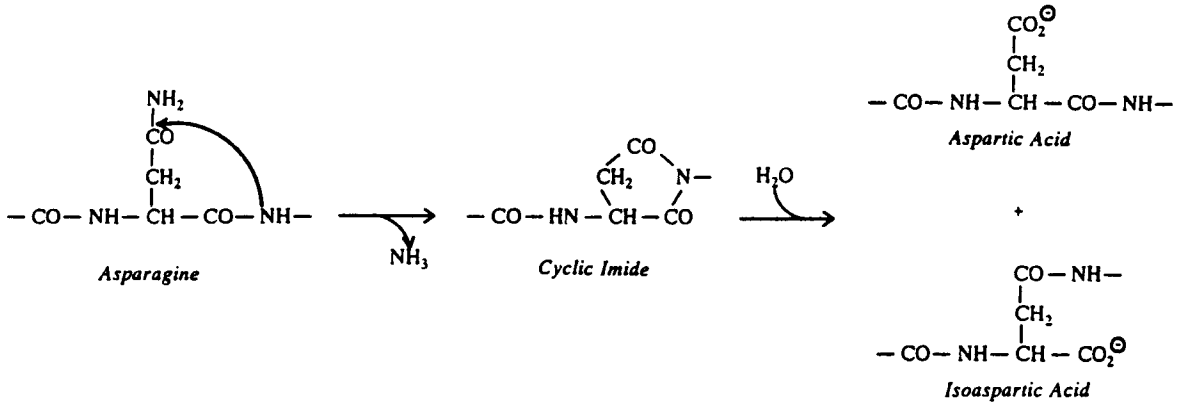

Fig. 1. Deamidation and isoaspartate formation in proteins proceeds from asparaginyl residues through a cyclic imide intermediate. *See* **ref. *12*** for mechanistic details.

shown to be sequence specific (half-life of deamidation ranging from 6–507 and 96–3409 d, respectively) depending on the identity of residue "X" ***(16,17)***.

Although some cases of protein deamidation may be caused by direct hydrolysis of amide linkages ***(14)***, this reaction is too slow to account for the deamidation of particularly labile peptide/protein bound asparagine residues. This suggests an intramolecular mechanism ***(12)*** in which, under neutral to basic solution conditions, the peptide bond nitrogen attacks asparaginyl carbonyl groups, causing ring closure with the concomitant release of ammonia. The resulting five-membered succinimide ring is unstable and susceptible to subsequent hydrolysis. This, in turn, leads to a mixture of α- and β-aspartyl residues (*see* **Fig. 1**). Under acidic conditions, deamidation is thought to proceed by direct hydrolysis, resulting in the formation of α-aspartyl residues alone ***(18)***.

By examining both the temperature and sequence dependence of the deamidation of a series of Asn containing hexapeptides at pH 7.4, Asn-Gly and Asn-Ser sequences were found to be particularly labile owing to decreased steric hindrance of succinimide formation by the C-terminal residue ***(19)***. For example, the Asn-Gly sequence manifested a half-life of only 1.4 d under physiological conditions. The authors also isolated and characterized the two main byproducts of the deamidation reaction, a succinimide intermediate (cyclic imide) and an isoaspartic acid residue. It was demonstrated that the succinimide product may racemize prior to hydrolysis (leading to the formation of D-amino acids) and that the overall ratio of iso-Asp to Asp residues after hydrolysis of the succinimide intermediate is typically 3:1. The corresponding Asp-Gly sequence can also form the succinimide product, although about 30-fold more slowly than Asn-Gly. A comprehensive examination of Asn and Asp hexapeptides showed a 232-fold sequence dependent range in the rate of succinimide formation under physiological conditions ***(20)***. The relative infrequency of detectable glutaminyl deamidations can be understood as a consequence of the instability of the corresponding six-membered glutarimide intermediate ***(12)***.

Are these sequence-dependent rates of Asn deamidation in peptides applicable to proteins with higher ordered structure? By comparing the rates of Asn deamidation in pentapeptides to the identical sequences in cytochrome C, Robinson and Rudd ***(15)*** concluded that secondary and tertiary structure does play an important role in dictating deamidation rates. More recently, comparisons of the deamidation rates of the above-mentioned Asn-Gly peptides to two Asn-Gly sequences in triosephosphate isomerase found about a 10-fold slower rate in the enzyme ***(21)***. Neutron diffraction studies showed that the sites of deamidation in protein crystals of trypsin do

not occur at the sites predicted from peptide studies *(22)*. Thus, protein tertiary structure, perhaps by dictating the flexibility of the polypeptide chain in the region of susceptible asparagine side chains, can either enhance or inhibit succinimide formation *(23)*. Nevertheless, many of the published examples of protein deamidation do, in fact, occur at Asn-Gly or Asn-Ser sequences *(12)*. Thus, although conformationally rigid regions of a protein molecule may inhibit deamidation at labile Asn residues, the presence of a sensitive Asn sequence in a particularly flexible region may enhance the susceptibility of this site to deamidation.

The detection of deamidation in proteins can be accomplished in a variety of ways based on either charge or molecular weight differences or by directly monitoring the formation of succinimide or isoaspartic acid residues. To illustrate the different strategies available to identify and characterize deamidation in proteins, examples using ribonuclease (RNase) as a model protein are summarized briefly. First, deamidation has been shown to contribute to the irreversible thermal inactivation of RNase as detected by purification of deamidated species employing preparative isoelectric focusing. The rate of formation of multiple deamidated RNase species was monitored by IEF and correlated well with the evolution of ammonia measured enzymatically *(24)*. Second, incubation of RNase under acidic conditions (0.1 *N* HCl, 30°C) led to protein deamidation with a resulting change in the enzyme's susceptibility to proteolytic cleavage *(25)*. Third, a specific deamidation event in RNase at Asn 67-Gly, in both native and unfolded enzyme, was monitored at a variety of temperatures and pH values by ion-exchange chromatography. It was found that this residue deamidates 30-fold more slowly in the folded form of the enzyme *(26)*. Finally, deamidated N67D-RNase recently was further purified into Asp and iso-Asp fractions using hydrophobic interaction chromatography; each species was then examined separately in terms of catalytic activity and renaturation kinetics *(27)*. Deamidation at Asn 67 to either Asp 67 or iso-Asp 67 lowered the catalytic activity of the enzyme toward a variety of substrates. Furthermore, as illustrated in **Fig. 2**, the Asp 67 derivative refolded at the same rate as the native enzyme, whereas the iso-Asp 67 form refolded 50% more slowly.

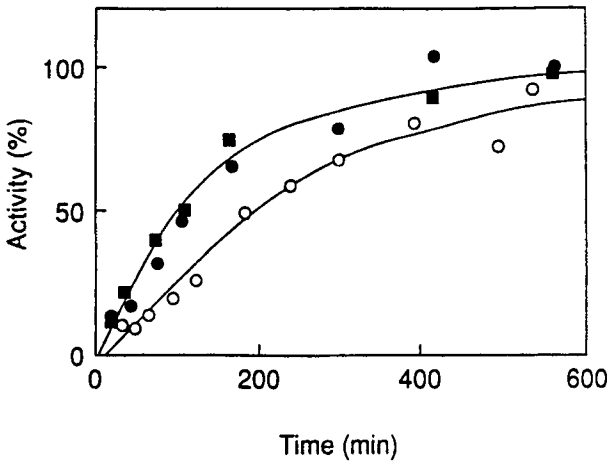

Fig. 2. Effect of deamidation on the renaturation of unfolded and reduced ribonuclease A (RNase): native RNase (Asn7) ■, deamidated RNase (Asp-67) ●, and (iso-Asp 67) ○. Experimental details in **ref. *27*** courtesy of American Society of Biochemistry and Molecular Biology.

Two other well-characterized examples of protein deamidation involve recombinant proteins under investigation as therapeutic agents, human growth hormone (rhGH), and the leech anticoagulant protein hirudin. Incubation of rhGH at pH 7.4, 37°C for up to 14 d resulted in the specific deamidation of Asn 149-Ser and isomerization of Asp 130-Gly *(28)*. Protein methyl transferase was used to label the isoaspartic acid residues in the altered rhGH with a radioactive methyl group. A combination of peptide mapping and subsequent amino acid analysis and mass spectroscopy was utilized to identify the specific deamidation sites in the purified peptides *(28)*. Capillary zone electrophoresis also was used to analyze deamidation in rhGH *(29)*. Interestingly, succinimide formation also was demonstrated in a lyophilized form of rhGH during storage at 45°C by using fast atom bombardment-mass spectroscopy to analyze both the intact protein and purified peptides from tryptic digestion (after resolubilization). An 18 U atomic weight difference corresponding to the loss of a water molecule was observed at the Asn 130 site in freeze-dried rhGH *(30)*.

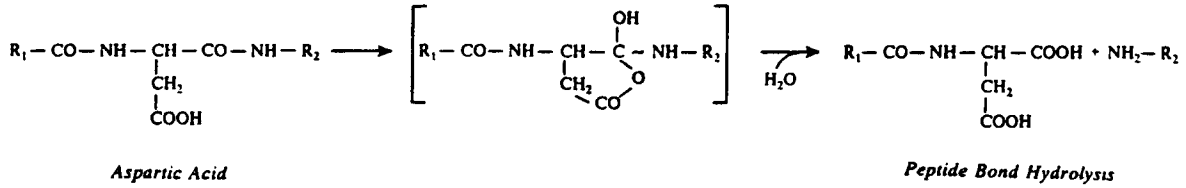

Fig. 3. Hydrolysis of peptide bonds at the C-terminal side of protein bound aspartic acid residues. *See* **ref. *35*** for mechanistic details.

Another example of the power of recent advances in bioanalytical technology to probe deamidation events is the use of anion exchange and reverse-phase (RP) HPLC to isolate two deamidated forms of recombinant hirudin (pH 9.0, 37°C). Acid-catalyzed carboxyl methylation was used to introduce a +15 U mass shift in each deamidated residue with subsequent detection by liquid secondary mass spectroscopy ***(31)***, resulting in the identification of two labile Asn-Gly sequences. During the purification of a mutant form of recombinant hirudin from yeast, a 1% impurity was identified in the final product by RP-HPLC. Further work separated this impurity into two peaks that were identified as succinimide-containing forms of hirudin corresponding to alterations of the two Asn-Gly sites described above ***(32)***. Identification was confirmed by both mass spectroscopy and by the presence of isoaspartic acid residues detected by sequence analysis (iso-Asp residues are resistant to Edman degradation).

3. Cleavage of Peptide Bonds

Three major mechanisms of peptide bond cleavage have been identified:

1. Preferential hydrolysis of peptide bonds at aspartic acid residues under acidic conditions;
2. At more physiological pH, C-terminal succinimide formation at Asn residues; and
3. Enzymatic proteolysis including autolysis.

The cleavage of a peptide bond obviously disrupts the linear sequence of amino acid residues within a protein chain. This covalent modification, however, may or may not affect the higher ordered structure of a protein and its biological activity. For example, there are numerous examples of both nonspecific hydrolysis or proteolysis leading to extensive protein degradation as well as specific proteolytic clips activating precursor forms of enzymes. Conversely, since the intramolecular interactions responsible for tertiary structure formation are sufficiently strong (cooperative), the introduction of a single intrachain clip in the polypeptide backbone may have little or no effect on a protein's structure or function.

Partridge and Davis ***(33)*** first reported the preferential release of aspartic acid residues after boiling a number of proteins in weak acid. This initial observation led to a series of papers examining hydrolysis of Asp-X sequences to determine whether this type of cleavage was sufficiently specific for sequence determination. When a series of proteins was heated in 0.03 *N* HCl (pH 2.0) at 105°C for up to 24 h and the amount of free Asp measured by amino acid analysis, it was found that Asp is preferentially cleaved and released at a rate at least 100 times greater than any other amino acid (for review, *see* **ref. *34***). The $t_{1/2}$ for this reaction (time required for the release of 50% of the total Asp and Asn residues) was 4 h for glucagon, 6 h for tobacco mosaic virus protein, 7.5 h for ribonuclease, and 24 h for insulin.

The preferential hydrolysis of a peptide bond at Asp residues is generally believed to occur at the C-terminal side of this residue in polypeptide chains, as shown in **Fig. 3**. The carboxyl group side chain of Asp catalyzes the cleavage reaction by acting as a proton donor at pH values below the pKa of the carboxyl group ***(35)***. The Asp-Pro bond is known to be particularly labile. Studies with model dipeptides (110°C in 0.015 *N* HCl) have shown that the Asp-Pro bond is 8–20 times more labile than other Asp-X or X-Asp sequences and over 100-fold less stable than the backbone

of peptides lacking Asp *(36)*. The greater acid lability of Asp-Pro peptide bonds is thought to be owing to either an enhanced α/β isomerization of aspartyl residues or the more basic nature of the proline nitrogen *(37)*.

The cleavage of the C-terminal Asp peptide bond at acidic to neutral pH has been demonstrated to contribute to the irreversible thermal inactivation of enzymes, such as lysozyme and ribonuclease *(38)*. For example, mechanistic studies have shown that Asp-X peptide bond hydrolysis accounts for 77% of the total loss of RNase enzymatic activity when heated at pH 4.0, 90°C with a half-life of about 7 h *(24)*. The hydrolysis at Asp residues recently was reported to contribute to the thermal degradation of more complex proteins, such as endoglucanase I and glyceraldehyde-3-phosphate dehydrogenase *(40)*. The long-term storage of recombinant human epidermal growth factor under moderately accelerated conditions (pH 3.0, 45°C) results in the partial cleavage of an Asp-Ser peptide bond *(41)*. Although the cleavages described above generally require moderately accelerated conditions of low pH and high temperature, these are, in fact, solution conditions under which protein folding and unfolding studies may often be conducted. Attempts to actually utilize the acid lability of Asp sequences have included construction of *E. coli* expression vectors encoding bovine growth hormone fusion proteins in which an Asp-Pro sequence was inserted between the two proteins. This permitted recovery of bGH from the fusion protein under acidic conditions *(42)*.

Cleavage of polypeptide chains can also occur under physiological conditions. Analogous to the deamidation reaction discussed in the previous section, succinimide formation at asparagine residues can potentially led to the spontaneous cleavage of polypeptide chains. In this case, the side-chain amide nitrogen attacks the peptide bond to form a C-terminal succinimide residue and a newly formed amino terminus *(12)*. This type of cleavage has been reported to occur in both model peptides *(18)* and in proteins (for review, *see* **ref. *12***). More commonly, contaminating proteases are often found to cleave recombinant proteins during both fermentation and purification *(43)*. Strategies to limit proteolysis include the addition of protease inhibitors, careful selection of cell host including protease negative mutants, sequence modification of susceptible sites in target proteins, and optimization of fermentation and purification conditions *(44)*. Storage of purified proteases under certain conditions may also lead to peptide bond cleavage (autolysis). The stabilization of subtilisin BPN against thermally induced autolysis by removal of a susceptible cleavage site through site-directed mutagenesis has been attempted with partial success *(45)*. A related approach was employed with tissue plasminogen activator (t-PA), a serine protease that contains an activation cleavage site at arginine 275. The product of the t-PA reaction, plasmin, clips t-PA resulting in conversion of the enzyme from a one-chain to a two-chain form. Site-directed mutagenesis was performed by replacing this Arg residue with the 19 other amino acids, thereby preventing the proteolytic clipping *(46)*. The "one-chain" mutant forms of t-PA had equivalent plasminogen activating activity in the presence of a fibrin(ogen) cofactor, yet diminished activity in the absence of the cofactor.

The detection and quantitation of peptide bond cleavage in proteins usually employ sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with gel scanning densitometry or measurements of the appearance of new amino and carboxy termini with subsequent sequence identification *(38)*. Particular caution is required during SDS-PAGE analysis. Sample preparation for electrophoresis often involves boiling proteins in an SDS solution, which, in turn, can cause artifactual peptide bond cleavage at Asp-X residues. For example, the preparation of human fibronectin *(47)*, β-galactosidase *(48)*, and fructose 1,6 biphosphatases *(49)* for SDS-PAGE has been reported to cause the appearance of multiple fragmentation byproducts. Recently, advances in both electrospray and laser desorption time of flight mass spectroscopy has permitted the direct detection of peptide bond hydrolysis products in both peptides and proteins. These techniques are emerging as the most accurate and sensitive meth-

ods to monitor such covalent changes *(50)*, since the resultant precise determination of the molecular weight of the peptide cleavage products allows their unambiguous identification.

4. Cystine Destruction and Thiol–Disulfide Interchange

Cystine residues (disulfides) are naturally occurring crosslinks that covalently connect polypeptide chains either intra- or intermolecularly. Disulfides are formed by the oxidation of thiol groups of cysteine residues by either thiol disulfide interchange or direct oxidation (*see* **Subheading 5.**). The probability of formation of a disulfide bond will depend on both the intrinsic stability of potential cystine residues compared to free cysteines and the conformation of the protein molecule *(51,52)*. Intracellular proteins usually lack such crosslinks and their atypical presence commonly reflects a role in an enzyme's catalytic mechanism or involvement in the regulation of its activity *(53)*. In contrast, extracellular proteins frequently contain disulfide bonds, probably reflecting the need for the increased stability of such proteins. The thermodynamic basis for this stabilization remains controversial, but cystine residues are believed to both directly stabilize the folded form of a protein and reduce the conformational entropy of the unfolded state *(53)*. Nevertheless, cystine residues are also "weak links" in protein molecules, since they are quite labile under certain conditions, especially when exposed to reducing potentials, heat, and alkaline pH.

The destruction of cystine residues in proteins have been shown to proceed by a base catalyzed β-elimination reaction in alkaline media (pH 12–13) *(54–56)*. The proton on polypeptide α-carbon atoms is relatively labile at high pH, since it is attached to two electron withdrawing groups (-CONH- and -NHCO-). This β-elimination results in the formation of two unstable intermediates, dehydroalanine and thiocysteine, as shown in **Fig. 4A**. The persulfide species breaks down into a variety of sulfur containing species, including hydrosulfide. Dehydroalanine is susceptible to nucleophilic attack, especially from the ε-amino group of lysine residues leading to the formation of the nonnatural crosslink, lysinoalanine. This same reaction can occur at neutral pH and elevated temperatures and has been shown to contribute to the irreversible thermoinactivation of ribonuclease and lysozyme at pH 6.0–8.0 and 90–100°C *(38)*. Another interesting example occurs in recombinant hirudin, a remarkably stable 65 amino acid protein with three disulfide linkages resistant to temperature, denaturant, and low pH induced unfolding. The combination of mildly elevated pH and temperature (pH 9.0, 50°C) leads to inactivation and loss of activity through β-elimination of the cystine residues and consequent formation of a mixture of atypical, unnatural crosslinks *(57)*.

Cystine destruction has been shown to be a quite general phenomena in a series of 12 different proteins heated a 100°C. Average half-lives of about 1 h at pH 8.0, 12 h at pH 6.0, and 6 d at pH 4.0 were encountered for these proteins *(58)*. In these studies it was also found that β-elimination leads to the generation of free thiol groups that can in turn catalyze a second degradative reaction, disulfide exchange *(24,58)*. For example, heat-induced thiol formation accelerated the release of glutathione from a synthetically prepared protein-glutathione mixed disulfide *(58)*. A similar inactivation pathway has been elucidated for the inactivation of insulin analogs during storage at pH 7.4 between 30 and 50°C *(59)*. This reaction was shown to be dependent on the conformational stability of insulin with the native state apparently protecting disulfides from chemical degradation (β-elimination and disulfide interchange).

Ryle and Sanger *(60)* first reported that model peptides such as cystine and oxidized glutathione undergo thiol catalyzed disulfide interchange at neutral to alkaline pH at 35°C. The reaction was accelerated by exogenous thiols and inhibited by reagents that block thiol groups. Thiols act as nucleophiles attacking the sulfur atom of the disulfide bond leading to mixed disulfide formation and subsequent scrambling of cystines as shown in **Fig. 4B** *(61)*. Since the reaction proceeds from the thiolate form, thiol–disulfide interchange is pH dependent with acceleration under alkaline

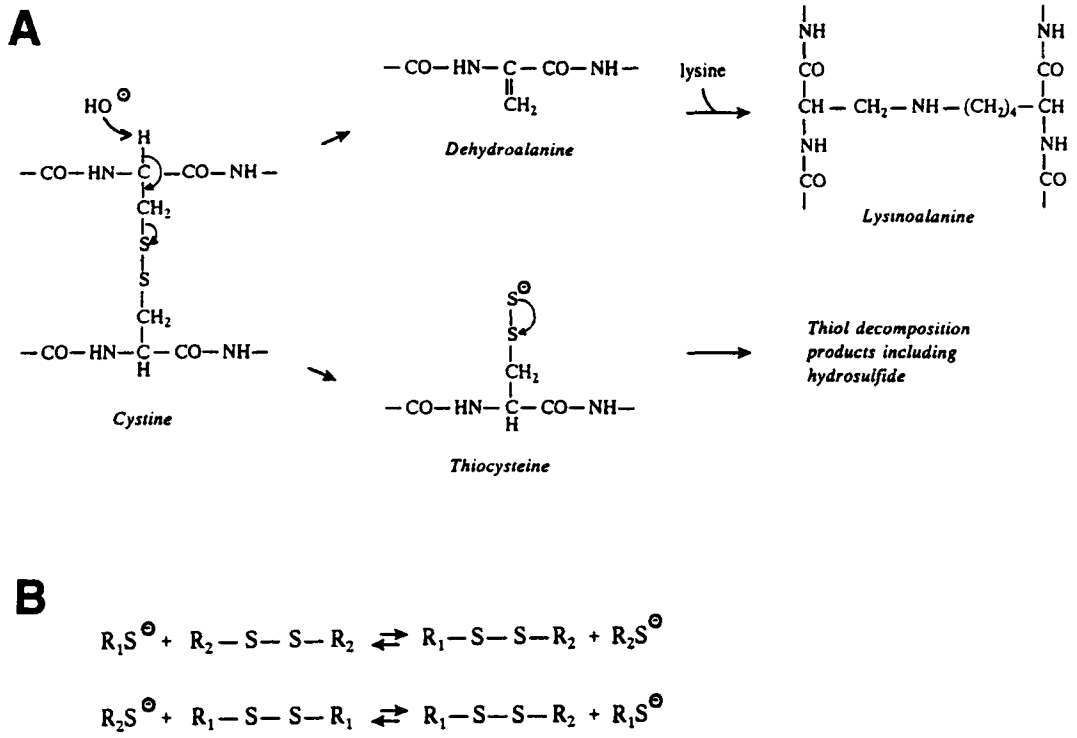

Fig. 4. Degradative reactions involving protein bound cystine residues. (**A**) Base catalyzed β-elimination, and (**B**) thiol catalyzed disulfide interchange reaction. *See* **refs. *55*** and ***61***, respectively, for mechanistic details.

conditions. Mixtures of reduced and oxidized thiols, such as glutathione, are commonly used to establish redox potentials that catalyze the reformation of protein disulfides during protein refolding experiments via thiol–disulfide interchange reactions.

In vivo, the intracellular concentration of thiols is quite high ***(62)***, present primarily in the form of reduced glutathione. This reducing environment is assumed to inhibit intracellular oxidation of protein cysteines to either protein disulfides or mixed disulfides formation with endogenous low molecular weight thiols. Mixed disulfide formation between protein cysteine residues and reduced glutathione in vitro can lead to enzyme inactivation ***(62)***. For example, the enzymatic activity of glutathione S-transferase is inhibited by biological disulfides, such as cystine and cystamine, which form active site mixed-disulfides that presumably sterically occlude the substrate binding region ***(63)***. A simple fluorescence-based assay has been used to monitor the concentration of mixed disulfides in α_1-protease inhibitor under a variety of redox conditions ***(64)***.

Both mixed disulfide formation and disulfide interchange can lead to altered structure and/or reduced conformational stability of proteins. For example, as shown in **Fig. 5**, mixed disulfide formation between lens crystallin proteins and glutathione (appearing in human cataracts) leads to the formation of modified proteins with reduced conformational stability as detected by guanidine hydrochloride unfolding experiments ***(65)***. Porcine ribonuclease inhibitor, a protein containing 30 cysteine residues, can undergo thiol–disulfide interchange leading to the formation of 15 nonnative disulfide bonds. This "all or nothing" reaction is proposed to be the result of a large conformational change induced by an initial thiol-disulfide interchange event that consequently results in the exposure of the other cysteine residues. The resultant protein has a much more open conformation than the native state as measured by gel filtration and susceptibility to proteolysis ***(66)***.

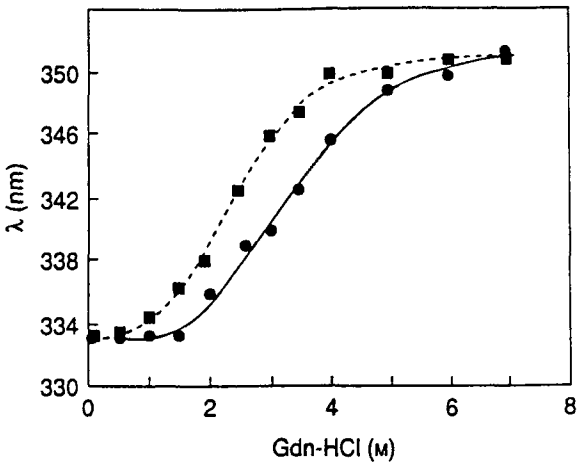

Fig. 5. Conformational destabilization of γ-crystallin owing to mixed disulfide formation with glutathione as detected by unfolding of the protein in guanidine hydrochloride: native γ-II crystallin (●) and GSSG-treated protein (■). Experimental details in **ref. *65*** courtesy of Academic Press.

The storage of proteins in an anhydrous state would be expected to slow down or eliminate these types of thiol disulfide interchange reactions. Despite this, freeze-dried proteins containing both disulfide bonds and reduced cysteine residues, such as bovine serum albumin, have been reported to undergo moisture-induced aggregation in the solid state owing to intermolecular disulfide formation ***(67)***. This, in turn, prevents resolubilization after rehydration.

5. Oxidation of Cysteine Residues

The relative stability of a reduced cysteine residue and its oxidized disulfide counterpart depends on the redox potential of a protein's environment ***(51,52)***. In vivo the electron donors and acceptors that interact with protein thiols and disulfides are primarily other thiols and disulfides (e.g., as reduced and oxidized glutathione). These compounds catalyze disulfide exchange reactions, as described in the previous section, resulting in the most thermodynamically favorable redox status of the protein's cysteine residues (free thiols vs disulfides). Similarly, in vitro protein refolding experiments require either redox buffers containing a mixture of oxidized and reduced thiol compounds to catalyze the oxidation of cysteine residues with the resultant reshuffling of disulfide bonds leading to the formation of native protein; or reducing agents, such as dithiothreitol to maintain cysteine residues in their active, reduced form.

Free thiol groups also spontaneously react with dissolved oxygen to form inter- and intramolecular disulfide bonds and monomolecular byproducts such as sulfenic acid as shown in **Fig. 6A** ***(61)***. This autooxidation reaction is catalyzed by divalent cations, such as copper and iron ions, proceeds at enhanced rates at elevated pH, and is potentially reversible on reduction. In fact, both thiol exchange and thiol oxidation reactions proceed via the thiolate ion that has a pKa of 8.0–9.0 for most biological thiols. It should be noted, however, that some protein thiols can exhibit pKa values that differ significantly from this range owing to local environmental effects. Each pH unit change toward the acidic decreases the oxidative reactivity of thiols by approximately an order of magnitude ***(52)***. Cysteine oxidation can also occur when protein solutions are exposed to other environmental conditions. Increased hydrostatic pressure has been shown to enhance oxidation of sulfhydryl groups in lactate dehydrogenase ***(68)***, whereas freezing of protein solutions concentrates solutes that may result in elevated levels of dissolved oxygen leading to an increase in oxidative reactions ***(69)***. Harsher oxidation conditions (e.g., performic acid) lead to the irreversible formation of cysteic acid (**Fig. 6A**).

The formation of disulfide bonds during the purification or storage of proteins containing naturally reduced cysteine residues often produces inactivation. For example, acidic fibroblast growth factor (aFGF), a potent mitogen for a variety of cells, normally contains three reduced cysteine residues and no disulfide bonds. As shown in **Fig. 7**, the copper catalyzed formation of an intramolecular disulfide bond inhibits the mitogenic activity of the protein, an effect reversed on reduction with dithiothreitol ***(70)***. Similar results have been demonstrated with intermolecular (dimer) formation in aFGF ***(71)***. Site-directed mutagenesis has been used to replace the cysteine residues with Ser and these aFGF mutants have

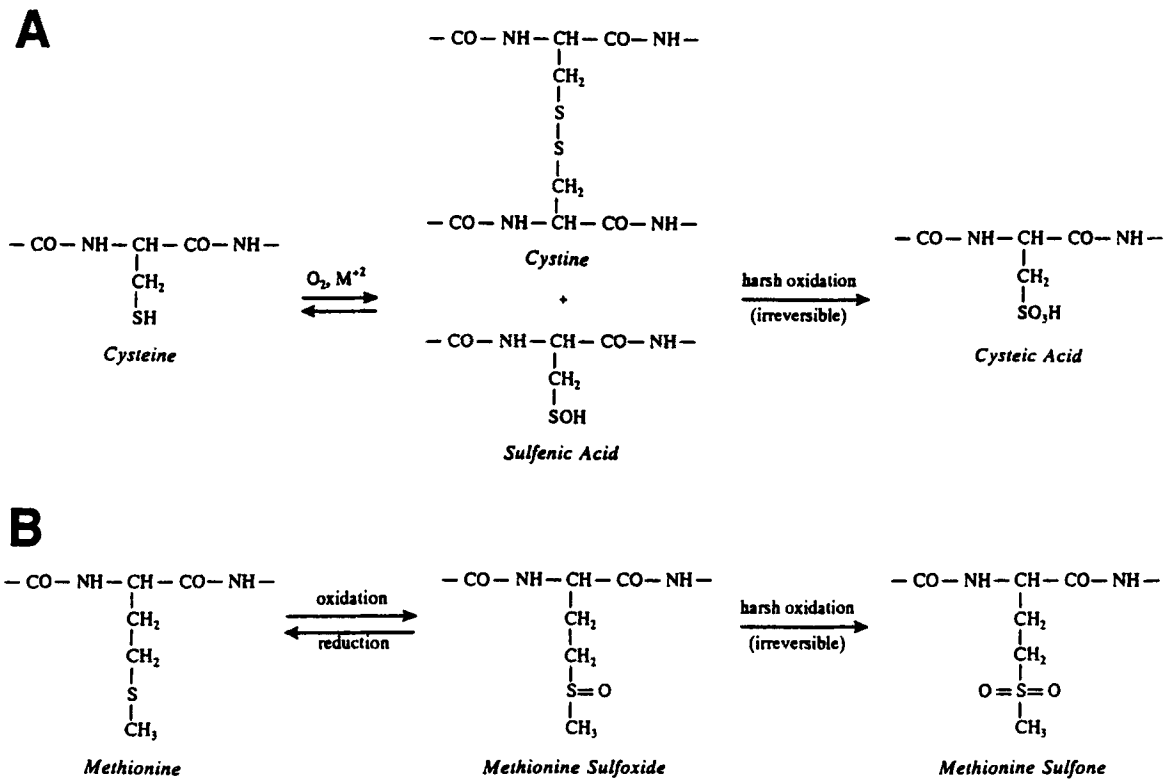

Fig. 6. Commonly encountered in vitro protein oxidation reactions: (**A**) conversion of cysteine residues to cystine, sulfenic acid, or cysteic acid, and (**B**) conversion of a methionine residue to its sulfoxide and sulfone counterparts. *See* **refs.** ***61*** and ***80***, respectively, for mechanistic details.

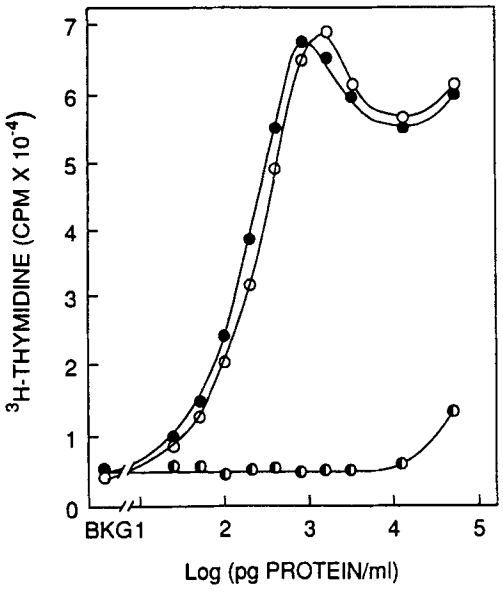

Fig. 7. Reversible inactivation of aFGF by oxidation of cysteine residues to cystine as measured by its ability to stimulate the growth of BALB/c3T3 cells monitored by incorporation of tritiated thymidine. (○) Native protein, (◑) oxidized protein, and (●) oxidized protein treated with the reducing agent dithiothreitol. Experimental details in **ref.** ***70*** courtesy of Harwood Academic Publishers.

increased stability and enhanced mitogenic activity *(72)*. Interestingly, binding of various polyanions to aFGF can also substantially reduce this susceptibility to oxidation, apparently by sterically occluding the most sensitive of the thiol groups *(73)*.

The irreversible thermal inactivation of both bacterial α-amylases and T4 lysozyme further illustrate the potential contribution of cysteine reactivity to protein inactivation. The irreversible thermal inactivation of microbial α-amylases is partially owing to autooxidation of the enzyme's single cysteine residue (pH 8.0, 90°C). Biochemical analysis of this oxidized cysteine residue

revealed that 30% was present as sulfenic acid, whereas 70% had formed intramolecular disulfides *(74)*. Wild-type T4 lysozyme contains two cysteine residues at positions 54 and 97 and no disulfide bonds. Inactivation of the wild-type enzyme has been shown by SDS-PAGE to be caused by cysteine oxidation to disulfide-linked oligomers *(75–78)*. On the introduction of a third cysteine residue at position 3 by site-directed mutagenesis, a disulfide bond is formed between Cys 3 and Cys 97, resulting in a protein with increased stability. In contrast, this disulfide containing mutant is now susceptible to thiol–disulfide interchange reactions with Cys 54. This protein can therefore be further stabilized by mutation of Cys 54 to Thr or Val. Despite the greatly improved stability of this double mutant, the enzyme still undergoes significant inactivation from a combination of noncovalent aggregation and chemical degradation *(78)*.

6. Oxidation of Methionine Residues

The oxidation of methionine residues has been associated with the loss of biological activity in a number of peptides and proteins *(79)*. As seen in **Fig. 6B**, the oxidation of methionine results in the conversion of this thioether to its sulfoxide counterpart *(80,81)*. This is a reversible reaction in which the methionine residue can be regenerated either by reducing agents or enzymatically. Harsher oxidative conditions cause irreversible formation of methionine sulfone. In vitro, proteins are commonly treated with dilute hydrogen peroxide (H_2O_2) solution or stronger oxidizers, such as chloramine T, to achieve methionine oxidation. In vivo, oxygen containing radicals, such as superoxide, hydroxyl, and H_2O_2, are generated in a variety of cells (e.g., neutrophils), leading to the oxidation of several amino acids, including methionine, with potential implications for various aging or disease-related processes *(79)*.

Oxygen radicals can also be generated in vitro by compounds commonly used in protein folding/unfolding studies. For example, small amounts of copper in the presence of glucose oxidizes a particular methionine residue in α_1-proteinase inhibitor *(82)*, whereas the autooxidation of reducing sugars can inactivate the enzyme rhodanese with a concomitant loss in sulfhydryl titer *(83)*. In addition, air oxidation of DTT can lead to H_2O_2 generation and subsequent protein oxidation *(84)*. Methionine oxidation can also occur during the purification and storage of proteins. For example, the fermentation and purification of recombinant antistasin from yeast *(85)* and the storage of lyophilized recombinant human growth hormone *(86)* result in the oxidation of specific methionine residues.

The oxidation of methionine residues in proteins does not necessarily cause either structural changes or loss of biological activity. There are numerous examples of sulfoxide formation of specific methionine residues in proteins, some leading to complete inactivation and others having little or no effect *(79)*. Perhaps the best studied example of the effects of methionine oxidation on the structure and activity of a protein is the enzyme subtilisin. Treatment of subtilisin with H_2O_2 leads to the formation of a methionine sulfoxide residue at position 222 near the catalytic site of the enzyme (Ser 221). This oxidation directly correlates with loss of enzymatic activity *(87)*. Site-directed mutagenesis has been used to replace this Met residue with nonoxidizable amino acids, resulting in dramatically improved resistance to oxidative degradation *(88)*. The three-dimensional structure of both native and peroxide inactivated subtilisin from *Bacillus amyloliquefaciens* has been determined by X-ray crystallography to examine the structural effects of peroxide oxidation on the enzyme *(89)*. In addition to Met 222, two of the remaining four Met residues were also observed to be partially oxidized as well as the hydroxyl groups of two of the enzyme's tyrosine residues. The oxidation of these Met and Tyr residues in subtilisin did not result in any global structural changes and, surprisingly, the reactivity of these sidechains with oxygen did not appear to correlate with their solvent accessibility.

7. Photodegradation of Proteins

Both ionizing and nonionizing radiation can cause protein inactivation. The effects of differ-

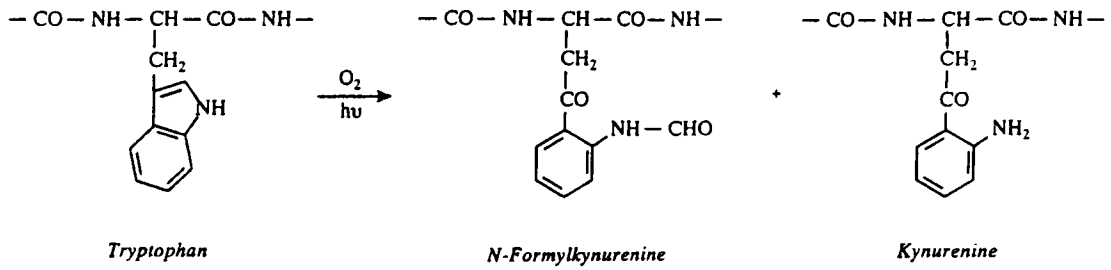

Fig. 8. Photooxidation of a tryptophan residue in proteins resulting in the formation of kynurenine (Kyn) and N-formylkynurenine (NFK). *See* **refs. *92–94*** for further discussion.

ent types of ionizing radiations (γ-rays, X-rays, electrons, α-particles) on a protein molecule (in both solid and solution states) have been examined in detail because of interest in the use of radiation as a potential sterilization technique in the food industry ***(90)***. Both direct effects (covalent changes, such as amino acid destruction or crosslinking) and indirect effects (radiolysis of water or buffer salts and subsequent protein alterations) have been extensively documented and recently reviewed ***(91)***. Nonionizing radiation, such as UV light, also may cause irreversible damage to protein molecules. These effects are of particular concern biologically in understanding the mechanisms of cataract formation and sunburn damage. In addition, protein unfolding/refolding studies frequently utilize UV/visible and fluorescence spectroscopy as methods of detection in which the potential adverse effects of incident light on proteins must be controlled and minimized.

The amino acids tryptophan, tyrosine, and cysteine are particularly susceptible to UV-A (320–400 nm) and UV-B (250–320 nm) photolysis ***(92–94)***. The absorption of photons leads to photoionization and the formation of photodegradation products through either direct interaction with an amino acid or indirectly via various sensitizing agents (such as dyes, riboflavin, or oxygen). Commonly observed photodegradation products in an aerated, neutral pH, aqueous protein solution include S–S bond fission, conversion of tyrosine to DOPA, 3-(4-hydroxyphenyl)lactic acid, and dityrosine as well as fragmentation byproducts, and the conversion of tryptophan residues to kynurenine (Kyn) and *N*-formylkynurenine (NFK) ***(92–94)***. The latter reaction is the most commonly observed and has been studied in the greatest detail (*see* **Fig. 8**). For example, model peptide studies have demonstrated that the rate of Gly-Trp photolysis is ten times greater than the corresponding reaction with Trp-Gly ***(95)***. The location of a Trp residue within the three-dimensional structure of a protein also influences its reactivity toward photolysis with increasing solvent exposure generally correlating with elevated reactivity ***(96)***.

The effect of potential photochemical modifications of tryptophan residues on the structure, stability, and activity of a protein must always be considered during protein unfolding/refolding studies. For example, the photooxidation of hydrophobic tryptophan residues causes significant crosslinking and conformational changes in lens crystallins ***(97)***. The UV irradiation of the *lac* repressor of *E. coli* leads to the photodegradation of Trp residues, resulting in both decreased fluorescence of the protein and loss of inducer activity ***(98)***. The chemical conversion of specific tryptophan residues (to Kyn and NFK) in lysozyme, ribonuclease T1, and a λ type immunoglobulin light chain have recently been examined to determine effects on the conformation and thermodynamic stability of these proteins ***(99)***. These modified proteins were found to have nearly identical overall structures as judged by CD and fluorescence spectroscopy, yet they displayed significantly reduced stability toward thermal and GuHCl induced unfolding (*see* **Fig. 9**). Moreover, the more hydrophobic the microenvironment of

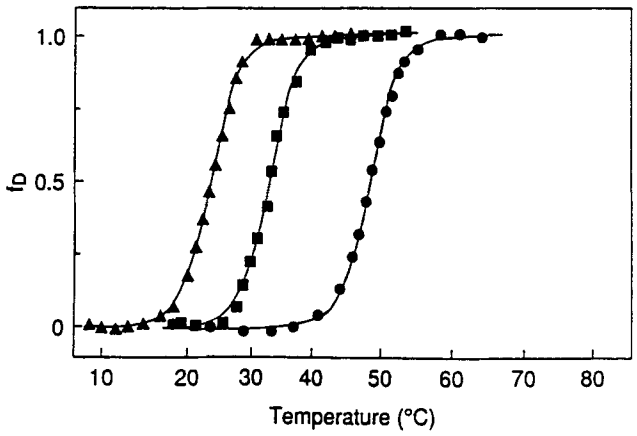

Fig. 9. Thermal unfolding of ribonuclease T1 and its derivatives as measured by circular dichroism: (●) Native protein (Trp 59), (■) Kyn-RNase T1, and (▲) NFK-RNase T1. Experimental details in **ref. *99*** courtesy of the American Chemical Society.

the tryptophan residue, the greater the effect of photolysis of this residue on decreasing the protein's stability. We have found that certain monoclonal immunoglobulins display remarkably large decreases in their intrinsic fluorescence emission intensities on exposure to UV light. This has been found to be a result of photolysis of single Trp residues in immunoglobulin hypervariable regions, with the large spectroscopic changes the result of the formation of NFK and Kyn residues that quench the overall fluorescence by a nonradiative energy transfer mechanism. Any protein molecule is potentially susceptible to photodamage during CD, UV, or fluorescent measurements. Thus, comparisons of consecutively collected spectra to assure stability of the CD, UV, or fluorescence signal (and hence aromatic residues) should be carried out routinely during such studies. In many cases, minimization of the intensity of the incident beam by reduction of slit widths and rapid scan rates combined with thorough degassing of solutions can prevent damage from photooxidation during optical measurements.

8. Glycation and Carbamylation of Protein Amino Groups

Sugars are frequently used as stabilizers of proteins during storage in solution or as lyophilized powders ***(100,101)***. Reducing sugars, however, can covalently react with protein amino groups (e.g., the ε-amino groups of lysine residues or the amino group at the *N*-terminus of polypeptide chains), which may lead to irreversible changes in the conformation and stability of proteins. When a reducing sugar, such as glucose, is incubated with proteins over long periods, the spontaneous formation of a Schiff's base between protein amino groups and glucose is often observed. Through a series of subsequent reactions known as the Amadori rearrangement, covalent adducts are then formed ***(102)***. This process is frequently referred to as the Maillard reaction or nonenzymatic browning (**Fig. 10A**). These Maillard adducts can further degrade to form so-called "advanced glycosylation end products" (AGEs), resulting in both protein crosslinking and the appearance of fluorescent byproducts. These glycation reactions are believed to be involved in degenerative processes in vivo. For example, the nonenzymatic browning of lens crystallins may play a role in cataract formation ***(103)***, and accumulation of AGEs is believed to correlate with the development of diabetic complications ***(104)***. The appearance of glucosylated hemoglobin (HbA1c) in diabetics is particularly well known ***(105)***. The kinetics of these glycation reactions as well as the analysis of various endproducts have been examined in detail with several proteins. For example, the glucosylation of ribonuclease A leads to the formation of dimers and trimers ***(106)*** and the frucation of bovine serum albumin to the formation of protein bound fluorescence ***(107)***. In the case of α-crystallins, glycation causes both conformational changes and destabilization of the protein against urea induced unfolding, as shown in **Fig. 11** ***(108)***.

Protein amino groups are also reactive with isocynate ions leading to the carbamylation of proteins ***(109,110)*** as shown in **Fig. 10B**. Urea is in equilibrium with isocynate ions. Therefore, protein unfolding experiments that employ high concentrations of this denaturant are always susceptible to this modification if proper precautions are not taken. These include minimization of the period of contact between urea and protein and

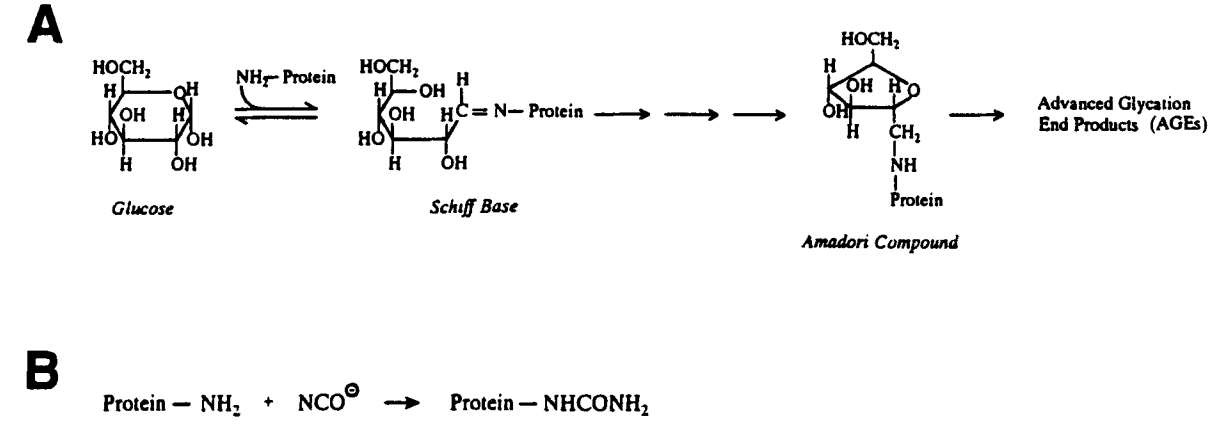

Fig. 10. Addition reactions at protein amino groups such as the ε-amino groups of lysine residues or the α-amino terminus of polypeptide chains. (**A**) Nonenzymatic browning reactions with reducing sugars such as glucose, and (**B**) carbamylation reaction with isocynate ions that are found in equilibrium with urea in aqueous solution. *See* **refs.** ***102*** and ***109***, respectively, for further elaboration.

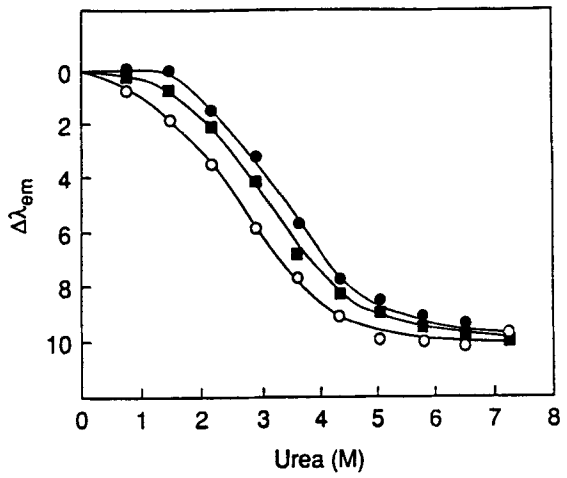

Fig. 11. Conformational destabilization of α-crystallins as a consequence of glycation of protein amino groups as measured by urea-induced unfolding: Native protein (●) and glucose 6-phosphate treated protein (■) and ribose treated protein (○). Experimental details are described in **ref.** ***108*** courtesy of Academic Press.

the use of freshly prepared urea solutions. Carbamylation is believed to play a role in cataract formation in certain disease states in vivo in which elevated levels of urea (and therefore isocynate) are present ***(103,111)***. Under conditions of neutral pH and moderate temperature, isocynate levels approach about 1% the amount of urea present ***(111)***. The kinetics of carbamylation of specific lysine residues in α-crystallin have recently been analyzed by a combination of IEF (isoelectric focusing) and fast atom bombardment mass spectroscopy ***(111)***. It was found that the protein's seven lysyl residues each had unique rate constants for carbamylation in which enhanced rates of individual lysine residues correlated with their greater solvent accessibility and, presumably, surface exposure. The carbamylation of several proteins, such as bovine growth hormone ***(112)***, insulin ***(113)***, and hemoglobin ***(114)*** has been shown to decrease their biological activities. The generation of specific antibodies against both glycated and carbamylated proteins has also been reported using modified proteins as immunogens ***(115,116)***. These antibodies may serve as the basis for alternative methods of detection for these protein amino group addition reactions to supplement the usual chemical approaches.

9. Conclusions

A variety of environmental conditions can lead to irreversible, covalent changes in protein structure. These alterations often adversely affect the conformation, stability, and bioactivity of a protein molecule. The purpose of this review is to heighten the reader's awareness of these potential reactions since many of the experimental conditions frequently used either during protein unfold-

ing/refolding studies or during the storage of protein solutions can themselves cause irreversible, covalent changes to protein structure. For example, the near-UV light employed in spectroscopic measurements (e.g., UV absorption, CD, fluorescence) may degrade aromatic residues, denaturants such as urea may cause carbamylation at lysine residues or at the *N*-terminus, and trace metal ions can autooxidize cysteine residues. Protein unfolding studies also are carried out frequently under nonphysiological conditions, such as high or low pH, in order to increase solubility and enhance reversibility of the folding process. Unfortunately, acidic pH can lead to hydrolysis of polypeptide chains (especially at Asp-Pro sequences), whereas alkaline pH can catalyze cystine destruction and asparagine deamidation. Compounds often used as protein stabilizers, such as sugars or reducing agents, can also react with proteins if the proper precautions are not taken. Thus, studies of what is assumed to be native protein may, in fact, actually be a mixture of modified and unmodified protein. Clearly, attempts to draw conclusions about fundamental processes in the folding/unfolding of proteins in such situations are fraught with significant hazards.

The conformation of a protein molecule can be a key factor affecting both the rate and extent of these covalent reactions. Thus, certain stages of a protein unfolding/refolding experiment are more "high risk" for covalent degradation of proteins. In the case of deamidation of asparagine residues and isoaspartate formation, increased flexibility of the polypeptide chain backbone greatly enhances the formation of the cyclic imide intermediate. Similarly, greater solvent accessibility (either through intrinsic surface exposure or partial unfolding) of cysteine, lysine, or tryptophan residues increases their lability toward oxidation, addition reactions, and photolysis, respectively.

Technological advances, such as mass spectroscopy and capillary zone electrophoresis, have greatly enhanced our ability to detect and quantitate the accumulation of covalently altered protein molecules. Recently, a monoclonal antibody against a specific isoaspartyl residue containing sequence in bovine growth hormone was generated ***(117)***. Clearly, the potential of developing monoclonal antibodies against a variety of chemical modifications in proteins now exists, both specific to individual proteins or particular modified residues. These antibodies could be used for the next generation of sensitive methods to detect covalent modifications of proteins. At the very least, we believe that any protein that has been subjected to significant stress in a protein folding/unfolding experiment should be examined by IEF. Although there are a few modifications that could go undetected by this method, most commonly observed covalent alterations do produce a change in charge that should produce a shift in the position of an IEF band. Importantly, lack of changes in biological activity and mobility on SDS-PAGE are not adequate for this purpose. Increasing use of capillary electrophoresis of native proteins should eventually enable this technique to be routinely used in place of IEF. Finally, mass spectroscopy, especially in the laser desorption time-of-flight format, with its 0.1–0.01% M_r accuracy, promises to become the method of choice to test for the presence of modified protein.

By understanding the causes and mechanisms of these protein modification reactions, strategies for minimizing their occurrence can also be implemented. We have found that it is extremely useful to carefully examine the amino acid sequence of a protein (if available) and identify amino acid "hot spots" before initiating protein unfolding/refolding studies. What strategies can be offered to minimize degradative, covalent reactions during the handling and storage of proteins? If appropriate, site-directed mutagenesis can be used to replace labile amino acid residues. For example, site-directed mutagenesis has been used to replace a labile Asn residue with Ser in recombinant interleukin-1α ***(118)*** and isoaspartate formation in recombinant human epidermal growth factor has been eliminated by the substitution of a particularly labile Asp residue with Glu ***(41)***.

Such time- and labor-intensive strategies, however, may not be necessary. One straightforward method is careful control of the temperature and pH of the protein solution. For example, the rate

of deamidation and isoaspartate formation as well as cysteine residue reactivity are enhanced under basic conditions. Elevated temperatures not only increase reaction rates but may increase protein flexibility and thus solvent exposure of labile amino acid residues. Since oxygen plays a role in both photolysis and autooxidation processes, these problems often can be minimized by avoidance of aerated solutions or removal of oxygen. Thus, the careful selection of experimental and storage solution conditions (including temperature, pH, and additives), based on the particular properties of the specific protein molecule under consideration, often can minimize successfully or even eliminate the occurrence of these deleterious processes. This suggests direct advantages in conducting folding/unfolding reactions at neutral pH and lower temperature using agents, such as guanidine-hydrochloride and chaotropic salts, to induce structural perturbation.

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