Protein Formulation and Delivery
Second Edition

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2

Chemical Considerations in Protein and Peptide Stability

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DEAMIDATION

Introduction

The deamidation reactions of asparagine (Asn) and glutamine (Gln) side-chains are among the most widely studied nonenzymatic covalent modifications to proteins and peptides (1–7). Considerable research efforts have been extended to elucidate the details of the deamidation reaction in both in vitro and in vivo systems, and a number of well-written, in-depth reviews are available (1–5,8,9). This work touches only on some of the highlights of the reaction and on the roles played by pH, temperature, buffer, and other formulation components. Possible deamidation-associated changes in the protein structure and state of aggregation also are examined. The emphasis is on Asn deamidation, since Gln is significantly less reactive.

Reaction Mechanism

The primary reaction mechanism for the deamidation of Asn in water-accessible regions of peptides and proteins at basic or neutral conditions is shown in Figure 1. For the present, discussion is confined to the intramolecular mechanism, uncomplicated by adjacent amino acids at other points in the primary sequence. Under alkaline conditions, the key step in the reaction is the formation of a deprotonated amide nitrogen, which carries out the rate-determining nucleophilic attack on the side-chain carbonyl, resulting in a tetrahedral intermediate and finally the formation of the five-member succinimide ring. For such a reaction, the leaving group must be

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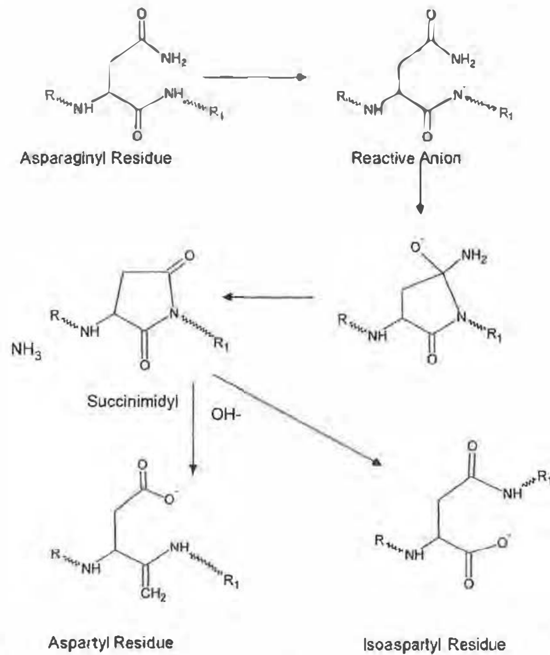

Figure 1 Proposed reaction mechanism for deamidation of asparaginyl residue. Note the formation of the succinimidyl intermediate and the two possible final products.

easily protonated, and in this case, it is responsible for the characteristic formation of ammonia (NH_3). The succinimide ring intermediate is subject to hydrolysis, resulting in either the corresponding aspartic acid or the isoaspartic acid (β-aspartate). Often, the ratio of the products is 3:1, isoaspartate to aspartate (10–12). In the case of acid catalysis (pH < 3), a tetrahedral intermediate is also formed, but breaks down with the loss of NH_3 without going through the succinimide (13–17). The reaction also appears to be sensitive to racemization at the α-carbon, resulting in mixtures of D- and L-isomers (10,13–15). The rate of degradation of the parent peptide in aqueous media often follows pseudo-first-order kinetics (16,17).

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A number of other alternative reactions are possible. The most prevalent reaction appears to be a nucleophilic attack of the Asn side-chain amide nitrogen on the peptide carbonyl, resulting in main-chain cleavage (10, 16, 18). This reaction (Fig. 2) is slower than that of cyclic imide formation and is most frequently observed when Asn is followed by proline, a residue incapable of forming an ionized peptide-bond nitrogen.

pH Dependence

Under conditions of strong acid (pH 1–2), deamidation by direct hydrolysis of the amide side-chain becomes more favorable than formation of cyclic imide (16,19). Under these extreme conditions, the reaction is often complicated by main-chain cleavage and denaturation. Deamidation by this mechanism is not likely to produce isoaspartate or significant racemization (16).

Under more moderate conditions, the effect of pH is the result of two opposing reactions: (*i*) deprotonation of the peptide-bond nitrogen, promoting

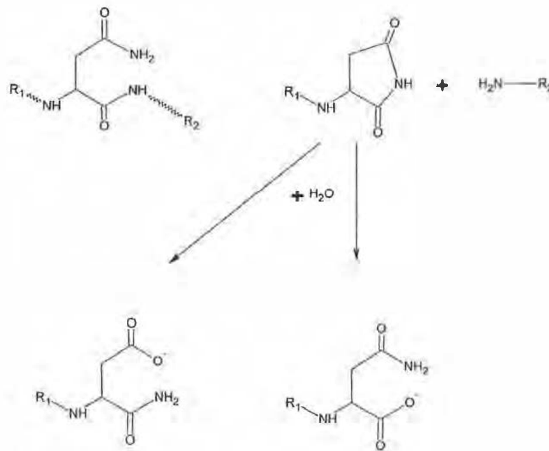

Figure 2 Proposed reaction mechanism for main-chain cleavage by asparaginyl residues.

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the reaction and (*ii*) protonation of the side-chain–leaving group, inhibiting the reaction. In deamidation reactions of short chain peptides uncomplicated by structural alterations or covalent dimerization (20), the pH-rate profiles exhibit the expected "V" shape, with a minimum occurring in the pH range of 3 to 4 (16). Computation studies by Peters and Trout (21) have been helpful in shedding light on the effect of pH. These authors have suggested that under mildly acidic conditions (3 < pH, 4), the rate-limiting step is the attack of the deprotonated nitrogen on the side-chain. The rate-limiting step at neutral pH is the hydrogen transfer reaction, while under basic conditions (pH > 7), it is the elimination of NH_2^- from the tetrahedral intermediate. Experimental studies have shown that the increase in rate on the alkaline side of the minimum does not strictly correlate with the increase in deprotonation of the amide nitrogen, indicating that the rate of reaction is not solely dependent on the degree of the peptide-bond nitrogen deprotonation (16,19). The pH minimum in the deamidation reaction measured in vitro for proteins may (22) or may not (23) fall in the same range as that of simple peptides. Overall pH-dependent effects may be modified by structure-dependent factors, such as dihedral angle flexibility, water accessibility, and proximity of neighboring amino acid side-chains (see section Peptide and Protein Structure).

Effect of Temperature

The temperature dependence of the deamidation rate has been studied in a variety of simple peptides in solution (16,24,25). Small peptides are easily designed to avoid competing reactions, such as oxidation and main-chain cleavage, and are thus useful to isolate attention directly on the deamidation rate. In solution, deamidation of small peptides tends to follow an Arrhenius relationship. Activation energies of the reaction do tend to show pH dependence, and a discontinuity in the Arrhenius plot is expected when the mechanism changes from direct hydrolysis (acid pH) to one of cyclic imide (mildly acidic to alkaline pH).

The deamidation rate of proteins also shows temperature dependence (23,26,27) under neutral pH. For deamidation reactions alone, temperature-associated rate acceleration in proteins may be due to enhanced flexibility of the molecule, allowing more rapid formation of the cyclic imide (28), or it may occur by catalysis by side-chains brought into the vicinity of the deamidation site (5).

The availability of water appears to be an important determinant in temperature-associated effects. In studies of lyophilized formulation of Val-Tyr-Pro-Asn-Gly-Ala, the deamidation rate constant was observed to increase about an order of magnitude between 40°C and 70°C (29). In contrast, in the solid state, the Arrhenius relationship was not observed. Further, the deamidation in the solid state showed a marked dependence upon the temperature when the peptide was lyophilized from a solution of pH 8, while little temperature dependence was observed when lyophilization proceeded from solutions at either pH 3.5 or pH 5. The authors related this temperature difference to changes in the reaction mechanism that may occur as a function of pH.

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Adjuvants and Excipients

The influence on deamidation by a variety of buffer ions and solvents has been examined. As pointed out by Cleland et al. (4) and reinforced by Tomizawa et al. (13), many of these additives are unlikely to be employed as pharmaceutical excipients for formulation, but they may be employed in protein isolation and purification procedures (30). Important clues to stabilization strategies can be gained from these studies. In the following, it is fruitful to keep in mind the importance of the attack of the ionized peptide-bond nitrogen on the side-chain carbonyl and the hydrolysis of the cyclic imide (Fig. 1).

Buffers

Buffer catalysis appears to occur in some but not all peptides and proteins studied (5). Bicarbonate (16) and glycine (12) buffers appear to accelerate deamidation. On one hand, the phosphate ion has been shown to catalyze deamidation, both in peptides and in proteins (12,13,16,31–34), generally in the concentration range of 0 to 20 mM. Capasso et al. (35,36) observed the acceleration of deamidation by acetate, carbonate, Tris, morpholine, and phosphate buffers only in the neutral to basic pH ranges. On the other hand, Lura and Schrich (37) found no influence on the rate of deamidation of Val-Asn-Gly-Ala when buffer components (phosphate, carbonate, or imidazole) were varied from 0 to 50 mM. A general acid–base mechanism by which the phosphate ion catalyzes deamidation was challenged in 1995 by Tomizawa et al. (13), who found that the rate of lysozyme at 100°C did not exhibit the expected linear relationship of deamidation rate on phosphate concentration. Although not linked to deamidation, it is worthwhile to note that at pH = 8 and 70°C, tris(hydroxymethyl) aminomethane buffer (Tris) has been shown to degrade to liberate highly reactive formaldehyde in forced stability studies of peptides (38).

Ionic Strength

The effects of ionic strength appear to be complicated and not open to easy generalizations. Buffer and ionic strength effects on deamidation are evident in proteins at neutral to alkaline pH (5). In selected peptides and proteins, the catalytic activity of phosphate has been shown to be reduced moderately in the presence of salts NaCl, LiCl, and Tris HCl (12,13). Of these salts, NaCl showed the least protective effect against deamidation (13).

In the peptide Gly-Arg-Asn-Gly at pH 10, 37°C, the half-life $t_{1/2}$ of deamidation dropped from 60 hours to 20 hours when the ionic strength was increased from 0.1 to 1.2 (22). However, in the case of Val-Ser-Asn-Gly-Val at pH 8, 60°C, there was no observable difference in the $t_{1/2}$ of deamidation when solutions without salt were compared to those containing 1 M NaCl or LiCl (12). Interestingly, for lysozyme at pH 4 and 100°C, added salt showed a protective effect against deamidation, but only in the presence of the phosphate ion (13).

In reviewing the data above, Brennan and Clarke (17) tentatively attributed the promotion of deamidation by elevated levels of ions to enhanced stabilization

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of the ionized peptide-bond nitrogen, promoting attack on the side-chain amide carbonyl. Other mechanisms would include disruption of tertiary structure in proteins that may have stabilized Asn residues, in some as-yet unknown fashion. That promotion of deamidation is observed in some cases of peptides, and inhibition in others does suggest rather complex and competing effects. Clearly, the stabilizing effects, when observed at all, are often at levels of salt too concentrated for most pharmaceutical formulations.

Solvents

The effect of various organic solvents on the rate of deamidation has not received much attention; it would be expected, however, that in the presence of a reduced dielectric medium, the peptide-bond nitrogen would be less likely to ionize. Since the anionic peptide-bond nitrogen is necessary in the formation of the cyclic imide, a low dielectric medium would retard the progress of the reaction and be reflected in the free energy difference for ionization of the peptide-bond nitrogen (17). Following this hypothesis, Brennan and Clarke (39) analyzed succinimide formation of the peptide Val-Tyr-Pro-Asn-Gly-Ala [the same peptide employed by Patel and Borchardt (16) in studies of pH effects in aqueous solution] as a function of organic cosolvent (ethanol, glycerol, and dioxin) at constant pH and ionic strength. The lower dielectric constant media resulted in a significantly lower rate of deamidation, in agreement with the hypothesis. It was argued that the similar rates of deamidation for different cosolvent systems of the same effective dielectric constant indicated that changes in viscosity and water content of the medium did not play a significant role.

The effect of organic cosolvents on deamidation in proteins is even less well characterized than that of peptides. Trifluoroethanol (TFE) inhibits deamidation of lysozyme at pH 6 and 100°C (13), and of the dipeptide Asn-Gly, but does not inhibit the deamidation of free amino acids. The mechanism of protection is not clear; direct interaction of the TFE with the peptide bond was postulated, but not demonstrated. An alternative hypothesis is that TFE induces greater structural rigidity in the protein, producing a structure somewhat resistant to the formation of the cyclic imide intermediate. Other, pharmaceutically acceptable solvents, ethanol and glycerin, did not exhibit the same protective effects as TFE on lysozyme.

Of course, in dosage form design, organic solvents such as TFE are not useful as pharmaceutical adjuvants. The effects of low dielectric may still supply a rationale for the solubilization of peptides in aqueous surfactant systems, where the hydrophobic region of a micelle or liposome could potentially enhance the stabilization of the Asn residues from deamidation. As pointed out by Brennan and Clarke (17), the results of experiments in organic solvents can have implications on the prediction of points of deamidation in proteins as well. For Asn residues near the surface of the protein, where the dielectric constant is expected to approach that of water, the deamidation rate would be expected to be high. For Asn residues buried in more hydrophobic regions of the protein, where polarities are thought to be

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more in line with that of ethanol or dioxane (40), reaction rates would be expected to be considerably slower.

Computational studies on the effects of solvent on the reaction were carried out recently by Catak et al. (41). They report that, in the absence of water, the overall activation energy barrier is on the order of 50 kcal/mol, and that this drops to a value of about 30 kcal/mol in the presence of water. In all, about three water molecules participate directly in the reaction, assisting in hydrogen transfer and in the cyclization outlined in Figure 1.

Polymers and Sugars

Considerable interest has developed in the stabilization of proteins and peptides in solid matrices, either polymeric or sugar based. In most solid polymer matrices, the primary role is to improve pharmacokinetic and pharmcodynamic properties of the active by modifying release characteristics and most studies are designed with this intention in mind (42). Sugars are usually employed as an aid to lyophilization of proteins, with the intent of maintaining the tertiary structure and preventing aggregation (43).

The state of the polymer and the activity of water appear to be critical factors in the stabilization of the peptide against deamidation. In general, the observed degradation rate constants exhibit the following rank order: solution > rubbery polymer > glassy polymer (38,44–46). However, this observation does not appear to be valid in every case (44). It has been proposed that up to 30% of a peptide may bind to polyvinylpyrrolidone (PVP) in the solution state, complicating the kinetic analysis (47).

Peptide stability in polymer matrices that are themselves also undergoing degradation provides a unique challenge. For example, it has been observed that PVP may form adducts with the *N*-terminus of peptides (48). Systematic studies of the deamidation of a model peptide in films of the copolymer polylactic–glycolic acid (PLGA) have shown that the reaction is the primary route of degradation only after longer storage times at higher water content (49). The delay in the onset of deamidation of peptide in PLGA may be related to the time necessary to establish an "acidic microclimate" that arises from the hydrolysis of the polymer (50). In support of this acid-catalyzed deamidation hypothesis in PLGA films, the reaction product, isoaspartate was not found.

Computational studies may supply additional insight. Computer simulations of the mobility of peptide, water, NH_3, and polymer in PVP matrix have been carried out by Xiang and Anderson (51). They observed that the diffusivity of water, NH_3, and peptide were between two and three orders of magnitude slower in PVP compared to aqueous solution. Importantly, the conformational dynamics of the peptide in the glassy polymer exhibited a higher energy barrier between states than seen for the peptide in water. Thus, two of the critical events in the process of deamidation, the conformational changes necessary to form the cyclic intermediate in the glassy polymer and the diffusion away of the NH_3 after release, are both slowed considerably in the solid state.

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The effect of sugars on the deamidation of a model peptide has been examined. At pH = 7, a solution of peptide in a 5% sucrose or mannitol reduced the deamidation rate to about 16% of that found in the absence of sugars (52). When stored in the solid state, the rate of reaction was even slower, although sucrose appeared to stabilize the peptide to a greater extent than did mannitol. It was observed that sucrose remained amorphous during the test period while mannitol crystallized, complicating the interpretation of the data (53). Cleland et al. (43) determined that 360:1 was the optimal sugar–antibody molar ratio necessary to inhibit aggregation and deamidation over a three-month period. Sugars sucrose, trehalose, and mannitol were able to stabilize the protein so long as less than 8.4% moisture was present.

Our understanding of the stabilization of peptides and proteins in polymer and sugar matrices is far from complete, and additional insight into the molecular mechanism might benefit from the bounty of studies carried out with small molecules in similar systems. Experiments must be designed carefully and interpreted with caution so as to clearly separate the solvent effects of water and perhaps even NH_3 on the reaction from the plastisizing effects on the matrix.

Peptide and Protein Structure

The ability to identify which Asn or Gln residues in a therapeutic protein or peptide may be vulnerable to deamidation would have great practical application in preformulation and formulation studies. The effects of various levels of structure—primary, secondary, and tertiary—are believed to be complex and varied. At present, only primary structure effects have been characterized in a systematic manner.

Primary Sequence

The primary sequence of amino acids in a peptide or protein is often the first piece of structural data presented to the formulation scientist. Considerable effort has been spent to elucidate the influence of flanking amino acids on the rates of deamidation of Asn and Gln residues. The potential effects of flanking amino acids are best elucidated in simple peptides, uncomplicated by side reactions or secondary and tertiary structure effects.

Effect of amino acids preceding Asn or Gln: In an extended series of early studies, Robinson and Rudd (24) examined the influence of primary sequence on the deamidation of Asn or Gln in the middle of a variety of pentapeptides. Mild physiologic conditions (pH 7.5 phosphate buffer at 37°C) were employed. A few general rules can be extracted from this work:

1. In practically every combination tested, Gln residues were less prone to deamidation than Asn. For the two residues placed in the middle of otherwise identical host peptides, the half-life of the reactions differed by a factor ranging from two- to threefold.

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2. In peptides Gly-X-Asn-Ala-Gly, steric hindrance by unionized X side-chains inhibits deamidation. The rank order of deamidation rate found was Gly > Ala > Val > Leu > Ile, with the $t_{1/2}$ ranging from 87 to 507 days. It remains unclear why bulky residues inhibit the reaction, but reduced flexibility of the sequence may be a factor. A similar effect was noted when Gln replaced Asn. In this case, $t_{1/2}$ ranged from 418 to 3278 days, in accordance with the diminished reactivity of Gln.
3. For the same host peptide, when the X side-chain was charged, the deamidation rate of Asn followed the rank order of Asp > Glu > Lys > Arg.

Effect of amino acids following Asn or Gln: Early experiments on dipeptides under extreme conditions indicated a particular vulnerability of the Asn-Gly sequence to deamidation (54). More recent studies of adrenocorticotropic hormone (ACTH)-like sequence hexapeptide Val-Tyr-Pro-Asn-Gly-Ala under physiologic conditions (55) have verified that deamidation is extremely rapid ($t_{1/2}$ of 1.4 days at 37°C). The formation of the succinimide intermediate is thought to be the basis for the sequence dependence (10) of deamidation. It is generally believed that bulky residues following Asn may inhibit sterically the formation of the succinimide intermediate in the deamidation reaction.

Steric hindrance of the cyclic imide formation is not the only possible genesis of sequence-dependent deamidation. The resistance to cyclic imide formation in the presence of a carboxyl-flanking proline peptide may be related to the inability of the protyl amide nitrogen to attack the Asn side-chain (10). The computational studies of Radkiewicz et al. (56) suggest that the effect of the adjacent residue may largely be attributed to electrostatic/inductive effects influencing the ability of the peptide nitrogen atom to ionize (as seen in Fig. 1). In the case of glycine, the inductive effect is insufficient to explain the results, and the authors argue that the ability of glycine to sample more conformational space compared to other amino acids may help stabilize the nitrogen anion. Experimentally, the replacement of the glycyl residue with the more bulky leucyl or prolyl residues resulted in a 33- to 50-fold (respectively) decrease in the rate of deamidation (10). Owing to the highly flexible nature of the dipeptide, the deamidation rate observed in Asn-Gly is thought to represent a lower limit.

In more recent studies, deamidation of Val-Tyr-X-Asn-Y-Ala, a peptide sequence derived from ACTH, was examined with different residues in both flanking positions (57). When X was histidine (and Y is glycine), no acceleration of deamidation was found relative to a peptide where X is proline. Placing a His following the Asn was found to result in similar rates of deamidation when X was phenylalanine, leucine, or valine. The rate when X was histidine was slower than that of alanine, cysteine, serine, or glycine. These results indicate that histidine does not have unique properties in facilitating succinimide formation. Of interest was the observation that histidine on the carboxyl side of the Asn did seem to accelerate main-chain cleavage products.

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Some of the general rules for peptides may also show higher levels of dependence on primary sequence. Tyler-Cross and Schrich (12) studied the influence of different amino acids on the adjacent amino end of the pentapeptide Val-X-Asn-Ser-Val at pH 7.3. For X= His, Ser, Ala, Arg, and Leu, deamidation rates were essentially constant and approximately seven times slower than the Val-Ser-Asn-Gly-Val standard peptide. Of special interest to the investigators was the observation of no difference in deamidation rates between those amino acids with and without β-branching (such as valine for glycine). This is in direct contrast to the findings of Robinson and Rudd (24) of 10-fold differences in deamidation for valine substitution for glycine in Gly-X-Asn-Ala-Gly, shown earlier. Under the mild alkaline conditions of Patel and Borchardt (16), Val-Tyr-X-Asn-Y-Ala, no difference in the deamidation rate constants was observed when proline was substituted for glycine in the X position.

Data mining: Data-mining approaches have been employed to formulate a semiquantitative means of predicting the effect of primary structure on rate of deamidation. Capasso (58) proposed the extrathermodynamic relationship shown in Equation 1:

$$\text{Log } k_1 = X_p + Z_{Asn} + Y_p \qquad (1)$$

Here k_1 is the observed rate constant for deamidation, X_p is the average contribution of the specific amino acid that precedes Asn, Y_p is the average contribution due to the amino acid that follows Asn, and Z_{Asn} is the value when both the preceding and following amino acids are glycine. Over 60 peptides were included in the database. As expected, the greatest influence on the deamidation rate in peptides was found to arise from the identity of the following amino acid. Some of the values for Y_p are listed in Table 1. As suggested previously, relative to the effect of glycine, bulky hydrophobic amino acids such as valine, leucine, and isoleucine

Table 1 Rate Constants Reported for the Reaction[a] of OH• with the Side Chains of Selected Amino Acids (101)

Amino acid	k (L/mole-s)[b]
Cysteine	4.7×10^{10}
Tyrosine	1.3×10^{10}
Tryptophan	1.3×10^{10}
Histidine	5×10^{9}
Methionine	8.3×10^{9}
Phenylalanine	6.5×10^{9}
Arginine	3.5×10^{9}
Cystine	2.1×10^{9}
Serine	3.2×10^{8}
Alanine	7.7×10^{7}

[a]Most values determined via radiolysis.
[b]The pH values of many of these studies have not been listed.

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appear to show the slowest deamidation rate, while the smaller, more polar histidine and serine show a rate closer to that of glycine. There do appear to be some discrepancies between these results and those mentioned earlier (12,16,57), in particular with respect to the experimentally observed effect of histidine. Clearly, different databases may give different results. At best, Equation 1 may be viewed as a first approximation for estimating deamidation rates in formulation studies.

Robinson et al. (59–61) have taken a different approach to mining by including means to account for the three-dimensional structure of the side-chains and by avoiding the use of data gathered in the presence of the known catalyst, phosphate buffer. A method has been proposed to estimate the deamidation reaction half-life at 37°C and pH = 7.4 based on the primary sequence (61). The extent to which this method may accurately predict the deamidation rate of peptides in pharmaceutical systems has not yet been rigorously tested experimentally, but if proven valid, it would supply a rather useful tool in guiding early formulation studies.

Secondary and Tertiary Structure

X-ray or nuclear magnetic resonance (NMR) data can provide a detailed map of the three-dimensional structure of the protein or peptide. The role of secondary and tertiary structures in intramolecular deamidation of proteins has been discussed by Chazin and Kossiakoff (62). It is beyond the scope of this work to present a comprehensive review of the details of deamidation reactions in specific proteins. Excellent reviews of a variety of specific proteins exist (6). For the most part, detailed mechanisms relating the secondary and tertiary structures of proteins to enhancement of rates of deamidation are not yet available.

Clear differences in the deamidation rates of some proteins are evident when native and denatured states are compared (13,63). Denaturation is thought to enhance main-chain flexibility and water accessibility (62). Sufficient conformational flexibility is required for the Asn peptide to assume the dihedral angles of $\Phi = -120°C$ and $\Psi = +120°C$ necessary for succinimide formation. In as much as such angles tend to be energetically unfavorable (64) in native proteins, it may be expected that Asn residues in the midst of rigid secondary structures, such as helices, may be resistant to deamidation. Other reactions, such as cross-linking might also give rise to rigid regions of the protein and enhanced resistance toward deamidation (65).

The direct influence of secondary structure on deamidation may be best understood in terms of hydrogen-bonding patterns that give rise to defined structures. The α-helix is characterized by the hydrogen-bonding of the main-chain carbonyl oxygen of each residue to the backbone nitrogen-hydrogen of the fourth residue along the chain. The resulting bond is close to the optimal geometry, and therefore maximal energy, for such an interaction (464). Hydrogen bonds in β-sheets are not of fixed periodicity as in the helix, but can exhibit comparable bond energies. Citing structural data for trypsin (66), Chazin and Kossiakoff (62) argue that strong main-chain hydrogen-bonding of the peptide nitrogen following Asn is an important factor in modulating deamidation. Since formation of the succinimide intermediate

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requires the peptide nitrogen to be free to attack the side-chain carbonyl, participation in a strong hydrogen bond by that nitrogen would inhibit the reaction. X-ray crystallography or NMR data may be helpful in identifying Asn residues in native structures likely to be protected by such a mechanism (62). Perhaps studies modeled along the lines of guest–host relationships would be helpful in elucidating further the influence of secondary structure on deamidation (67).

Effects of Deamidation on Secondary and Tertiary Structure

The effects of deamidation on the secondary and tertiary structure of the reaction-product protein have been difficult to generalize (7). In 1994, in an extensive and detailed series of studies, Darrington and Anderson showed that deamidation strongly influences the noncovalent self-association (68) and covalent dimer formation (68,69) of human insulin. The noncovalent dimer formation of triosephosphatase (70) is inhibited by deamidation, probably by charge repulsion arising from the resulting additional anionic charges present in the hydrophobic faces of the monomers.

Deamidation in concentrated solutions of food proteins tends to show increased viscosity, possibly due to enhanced charge interactions between formerly uncharged portions of the protein molecule (63). The isoelectric point of the deamidated molecule is shifted toward lower values, possibly resulting in the modified potential for adsorption to solid surfaces (71). Foamability of protein solutions subject to deamidation is greatly enhanced, probably because of partial unfolding (63).

Deamidation can destabilize a protein, making thermal (70) or chemical (13) denaturation more likely. Folding patterns may be influenced (72,73), and changes in secondary structure can result (70). Other proteins appear to be resistant to structure alterations secondary to deamidation (74,75).

OXIDATION

Introduction

Oxidation has been identified as another of the major degradation pathways in proteins and peptides and can occur during all steps of processing, from protein isolation to purification and storage (76,77). A change in the biological activity of a therapeutic agent potentially can arise from an altered enzymatic activity, inhibited receptor binding properties, enhanced antigenicity, or increased sensitivity to in vivo proteases. In some instances, biological activity is completely or partially lost upon oxidation, while in other instances, no effect on bioactivity is observed. The molecular mechanism of altered bioactivity often comes about either by oxidation of a critical residue at or near the enzyme active site or receptor binding site, or by a dramatic change in the structure of the protein upon oxidation. At present, no general rules are evident to predict with certainty all the effects of oxidation on the biological activity of a particular protein.

The chemistry of autoxidation (i.e., oxidation, not enzyme- or radiation-catalyzed) in nonprotein drug molecules has been reviewed (78,79). There are

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three main steps that make up any free radical chain reaction oxidation mechanism, namely: initiation, propagation, and termination. In the initiation step, free radical generation is catalyzed by transition metal ions, light energy, or thermal energy. Once initiated, oxidation reactions propagate by chain reactions of organic substances with reactive oxygen species such as singlet oxygen, hydroxyl, and peroxyl radicals. The propagation steps are either hydrogen atom abstraction or addition to olefin. In the termination step, free radicals, both alkyl and reactive oxygen, are consumed without producing further radicals among the products. For the purposes of pharmaceuticals, it is important to emphasize the role of both trace metal ions and dissolved oxygen in accelerating oxidation (76,80).

Oxidation in Pharmaceutical Proteins and Peptides

In living systems, a variety of well-characterized reactive oxygen species are produced (81,82). In pharmaceutical formulations, identifying a single oxidation initiator is often difficult, since a variety of initiation possibilities exist, such as photochemical (83,84), metal ion catalyzed (85,86), and high energy γ-radiation (85). Even something as seemingly simple as sonication may promote the generation of reactive oxygen species (87). It has been convincingly shown that the extent of protein oxidation, and subsequent loss of biological activity, exhibits strong dependence upon the oxidation system employed (84,88–90).

In pharmaceutical proteins, transition metal ion catalysis of oxidation has received the lion's share of attention (89,91,92), while much less attention has been devoted to light energy and thermal energy (83,84).

Metal Ion Catalysis of Oxidation

Because of their importance in biological systems, a variety of metal ion–catalyzed oxidation systems have been identified and cataloged (85). Since the metal ion–catalyzed systems tend to be amenable to laboratory manipulations, they have been employed in stability studies (89,91,92). More importantly, trace levels of metal ions known to initiate oxidation are often present as contaminants in pharmaceutical systems (76), making an understanding of metal ion catalysis highly relevant to the job of formulation stabilization.

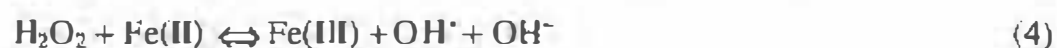
Iron(II) and copper(II) salts, in the presence of molecular oxygen and water, will slowly oxidize to form $O_2^{\bullet-}$ (superoxide radical) by Equation 2.

$$Fe(II) + O_2 \Leftrightarrow Fe(III) + \bullet_2^{-} \quad (2)$$

The superoxide radical is not stable at neutral pH and undergoes dismutation to form hydrogen peroxide by Equation 3.

$$2O_2^{\bullet-} + 2H+ \Leftrightarrow H_2O_2 + O_2 \quad (3)$$

Hydrogen peroxide reacts further to produce hydroxyl radicals ($OH^{\bullet}$) by Equation 4.



$$H_2O_2 + Fe(II) \Leftrightarrow Fe(III) + OH^{\bullet} + OH^{-} \quad (4)$$

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Hydroxyl radicals are capable of abstracting hydrogen atoms with bond energies less than 89 kcal/mol (93–95), producing a carbon-centered radical by Equation 5.

$$OH^{\bullet} + RH \Leftrightarrow H_2O + R^{\bullet} \quad (5)$$

In the presence of oxygen, the carbon-centered radical forms the organic radical $ROO^{\bullet}$. $ROO^{\bullet}$ is capable of entering a variety of chain-reaction propagation and termination reactions (91). Overall, at least four different reactive oxygen species, each able to oxidize pharmaceutical proteins, may be produced. In solutions of free amino acids, oxidation by $OH^{\bullet}$ shows a strong dependence on bicarbonate ion concentration (85,96). It has been suggested that the bicarbonate ion may be required to interact with the amino acid and Fe(II) to form a hybrid complex.

Site-Specific Metal Ion–Catalyzed Oxidation

Radiolysis studies have shown that all amino acid side-chains are vulnerable to oxidation by reactive oxygen species. The same oxygen radicals, when produced by metals (Eqs. 2–5), tend to attack preferentially only a few amino acid residues, most notably His, Met, Cys, and Trp. In addition, metal ion–catalyzed oxidation of proteins can show relative insensitivity to inhibition by free radical scavenger agents (91,92). These observations have led to the hypothesis that metal ion–catalyzed oxidation reactions are "caged" processes in which amino acid residues in the immediate vicinity of a metal ion binding site are specific targets of the locally produced reactive oxygen. Schoneich and Borchardt have discussed the following reaction (92):

$$D\text{–}Fe(II) + O_2 + H^+ \rightarrow D\text{–}Fe(III)\text{--}OOH \quad (6)$$

D is some binding ligand, such as a buffer species, peptide, or protein. By this mechanism, any amino acid residues capable of forming a metal ion binding site are potential sources of reactive oxygen species. Since reaction of the oxygen radical usually occurs in the immediate region of its production before escape into the bulk solution by diffusion, free radical scavengers are unlikely to be efficient formulation protective agents (92). It has been suggested that the terminal hydroxyl group of serine, the free carboxyl groups of aspartic and glutamic acids, the imidazole ring of histidine, and the free amino or free carboxyl groups of *N*-terminal and *C*-terminal (respectively) residues participate in binding metal ions to proteins (96). Further, since a metal ion binding site may be formed by appropriate residues upon folding of the protein molecule, these amino acids need not be adjacent to each other in the primary sequence.

Oxidation by Hydrogen Peroxide Addition

Addition of hydrogen peroxide has been employed as a means to study oxidation of proteins (88,97,98), the advantage being that the concentration and identity of the initiating oxidant is known. In some instances, hydrogen peroxide has been shown to be an oxidant specific for methionine (99), while in other instances, oxidation of cysteine and tryptophan residues also occurs (100). Hydrogen peroxide

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is thought to oxidize only residues easily accessible on the surface of the folded protein, but more recent evidence suggests oxidation of both surface and buried residues (99). It has been proposed that *t*-butyl hydroperoxide may be a highly specific oxidizer of surface-localized methionine residues (99).

A highly detailed mechanism of the oxidation by hydrogen peroxide of the amino acid cysteine to the disulfide has been recently published (98), showing the formation of cysteine sulfenic acid as an intermediate. These authors proposed a two-step nucleophilic reaction where the thiolate anion attack on the neutral hydrogen peroxide is the rate-determining step. Effects of buffer pH, temperature, and ionic strength were all included in the model. Well-controlled studies such as this will go a long way to elucidating details of the molecular mechanism(s) of oxidation.

Specific Amino Acid Side-Chains

Overview of Amino Acid Oxidation

First-order rate constants for the reaction of OH with the side-chains of selected amino acids have been listed (101) and representative values are shown in Table 1. As a free amino acid, cysteine is the most sensitive to reaction followed closely by the aromatic side-chains. Overall, the reaction rate constants vary less than three orders of magnitude, with the higher values approaching the diffusion limit. Although these values are specific for reactions with free amino acids and thus do not take into account effects due to accessibility, they may be useful as early estimates of the sensitivity of a peptide to OH. Not listed in Table 1 are the reported values for the reaction of OH with selected small peptides (101). In general, the value for the reaction rate constant of a peptide appears to be close to that of the most reactive individual amino acid (101).

One of the most damaging reactions of oxygen radicals is that of hydrogen abstraction from the peptide backbone, in particular at the α-carbon (102,103) As shown in Figure 3, in the presence of O_2, a peroxyl radical is formed that can convert to an imine, followed by hydrolysis of the backbone (102,103).

Methionine

Methionine has been identified as an easily oxidized amino acids in proteins (Table 1), and oxidation of this residue has received considerable attention. Oxidation deprives methionine of its ability to act as a methyl donor, which will influence the bioactivity of proteins dependent on that function (104). The reaction product of methionine oxidation is the corresponding sulfoxide and, under more strenuous oxidation conditions, the sulfone (Fig. 4). These are not the only possible reaction products, but they are usually the first to appear.

Not surprisingly, mechanisms of oxidation of methionine appear to be highly dependent on the reactive oxygen species under consideration (84). Peroxide (105), peroxyl radicals (106), singlet oxygen (105), and hydroxyl radical (91) have all been shown to oxidize methionine residues to sulfoxides and other products. The identity of major oxidizing species present in these solutions remains a matter of

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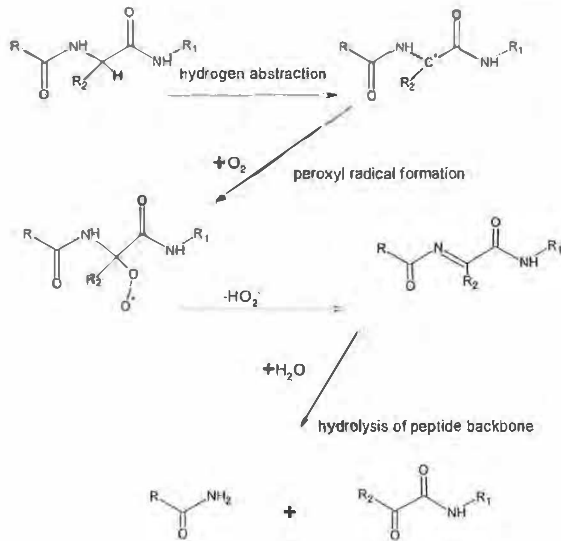

Figure 3 Involvement of peroxyl radical in the hydrolysis of peptide backbone.

controversy (107,108). The reaction mechanisms for proteins in pharmaceutical systems are incomplete, because not all products and intermediates are known.

Oxidation of methionine in recombinant human relaxin: *Photocatalyzed oxidation.* A series of papers spanning the 1990s studied methionine oxidation initiated by light (84), hydrogen peroxide (88), and ascorbic acid–Cu(II) (89) in recombinant human relaxin. Upon exposure to light of an intensity of 3600 candles for 5 to 17 days, both methionine residues, Met-B4 and Met-B25, located on the surface region of the B-chain were oxidized to the sulfoxide derivative (84). The identity of the reactive oxygen species formed upon exposure to light was not reported, but peptide mapping results suggest a wide variety of reaction products.

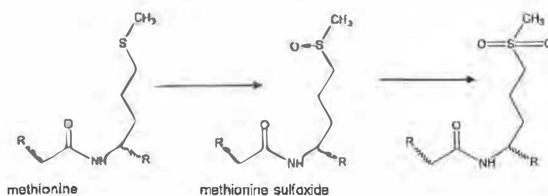

Figure 4 Oxidation of methionine first to the sulfoxide and then to sulfone derivatives.

Hydrogen peroxide. In the presence of added hydrogen peroxide, the methionines (Met-B4 and Met-B25) were the only residues of relaxin to be oxidized (88). Three products were isolated, the monosulfoxide for each methionine and the corresponding disulfoxide. The reaction rate was independent of pH (range 3–8), ionic strength (0.007–0.21 M NaCl), or buffer species (lactate, acetate, Tris). Interestingly, the rate of reaction of the two methionine groups differed, with oxidation at Met-B25 being more rapid than at Met-B4. The oxidation rate of Met-B25 was equivalent to that observed for free methionine and for methionine in a model peptide of the relaxin B-chain (B23–B27). The reduced rate of oxidation at the solvent-exposed residue Met-B4 relative to Met-B25 suggests that accessibility of the residues to H_2O_2 may play a role in the reaction.

Pro-oxidant system ascorbic acid–Cu(II). Contrary to the results observed in the presence of hydrogen peroxide, in the presence of the pro-oxidant system ascorbic acid–Cu(II), a pH-dependent precipitation of relaxin was observed (89). Approximately 80% of protein was lost from solution within 25 minutes at pH 7 to 8. Chromatographic results indicated that the aggregate was not held together by covalent forces. In a second significant aspect of the study (88), in the presence of ascorbic acid–Cu(II), investigators observed oxidation of histidine and methionine (89). One final important difference is that Met-B4 was oxidized preferentially over Met-B25. All these differences are consistent with the conclusion that the oxidant system employed for in vitro studies can have a major impact on the results. Clearly, the issue of identifying the radical species responsible for oxidation of methionine, or any other residue, is of primary importance in setting down complete reaction mechanisms.

Methionine oxidation studies with model peptides: As has been pointed out, development of a molecular-level understanding of oxidation in protein drug delivery systems has been hampered by a lack of characterization of the reaction mechanism and the products. A trail-blazing work by Li and coworkers (91,92,109) has begun for addressing the much-needed mechanistic description of the effects of pH and primary sequence on oxidation pathways of methionine in simple model peptides. These authors have primarily employed the metal ion-catalyzed pro-oxidant system and a series of simple methionine-containing

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peptides. Considerable efforts have been expended with specific radical scavengers to identify the reactive oxygen species responsible for oxidation.

Buffers and pH: Using the pro-oxidant systems of dithiothreitol/Fe(III) to generate reactive oxygen species, oxidation of methionine in Gly-Gly-Met, Gly-Met-Gly, and Met-Gly-Gly was studied as a function of pH. The degradation rate followed first-order kinetics with respect to peptide, while mass balance comparisons showed that sulfoxide was not the terminal degradation product. The rate of loss of the parent peptide did not vary with pH in the range 6 to 8.1. The rate of loss was observed to accelerate with pH beyond this range.

Li et al. (109) found that the second-order rate constants for the degradation of His-Met in the ascorbic acid–Fe(III) pro-oxidant system show a maximum at pH 6.4. The appearance of a maximal pH was attributed to competing effects of pH on ascorbic acid. Deprotonation of ascorbate at a higher pH ($pK_1 = 4.1$) facilitates electron donation to Fe(III) and accelerates the initiation reaction, while at the same time, ascorbate becomes a better oxygen radical scavenger, inhibiting the reaction. The buffer species also seems to play a role in the kinetics of degradation. In buffers of equal ionic strength, methionine oxidation was faster in the presence of phosphate than in the presence of Tris or HEPES. Phosphate buffers may facilitate the electron transfer from Fe(II) to oxygen, promoting the reaction (109). Buffer species such as Tris or HEPES have a weak affinity for metal ions (110) and result in methionine oxidation reaction rates that are somewhat less than that of phosphate. Tris and HEPES have also been reported to be scavengers of hydroxyl radicals (111), which would be expected to further inhibit reactions in which the hydroxyl radical is the primary reactive oxygen species. In temperature studies, the energy of activation was found to be 23.9 ± 2 kJ/mol, but it is unknown whether this characterizes the formation of the oxidizing species, the oxidation of methionine, or both.

Primary sequence: Li et al. (109) also studied the effect of primary structure on methionine oxidation. When in a terminal position, Met-Gly-Gly or Gly-Met-Met, the first-order degradation rate constants are greater than that of the mid-position Gly-Met-Gly. The inclusion of histidine in His-Met greatly accelerates the degradation of methionine. The greatest degradation rates are observed in His-Gly-Met and His-Pro-Met, where methionine is separated by one residue from histidine. Even His-Gly-Gly-Gly-Gly-Met shows enhanced degradation rates (by a factor of 5) compared to Gly-Gly-Met. Whether this is related to the metal ion binding and localized oxidation is not yet known with certainty. The authors do note that the degradation products of these reactions have not been characterized, and complete reaction mechanisms are not yet available.

Histidine

Histidine is also highly susceptible to oxidation, either by photocatalyzed or by metal ion–catalyzed mechanisms. Photooxidation of proteins in vivo has been extensively studied (112,113). Photosensitizing agents such as methylene blue

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(114) or rose bengal (115) are required for photooxidation to take place via the production of singlet oxygen ($^{1}O_2$). A cycloperoxide ring is produced by the addition of $^{1}O_2$ to the imidazole ring of histidine (116). The kinetics of photoreactions are often very complex (117), being further complicated by issues of histidine accessibility to solvent and $^{1}O_2$ (118) as well as simultaneous metal-catalyzed oxidation (119). A variety of products are produced, including the amino acids aspartic acid and Asn (76,120).

From the standpoint of pharmaceutical formulations, recent evidence suggests that the oxidation products of ascorbic acid (a frequently employed antioxidant) may be potent photosensitizing agents, enhancing histidine oxidation in proteins. Ortwerth et al. (119) reported $^{1}O_2$ concentrations in the millimolar range and H_2O_2 in the micromolar range after one hour of irradiation with ultraviolet light in the presence of dehydroascorbate and diketogulonic acid (by-products of ascorbic acid oxidation). Complete protection against photooxidation can be attained by protection from light or removal of all dissolved oxygen gas (112).

There has been some study of the effect of primary structure on the photooxidation of histidine. Miskoski and Garcia (112) found little difference in the rate of photooxidation of histidine as the free amino acid and in dipeptides His-Gly and Gly-His. Changing the solvent to acetonitrile/water (1:1) resulted in an order-of-magnitude decrease in the rate of oxidation in all three substrates, which is suggestive of polarity effects on the rate of reaction.

Histidine appears to be particularly sensitive to transition-metal-catalyzed oxidation, presumably because it often forms a metal binding site in proteins (121). Fenton chemistry at the bound metal ion (such as that as in Eq. 6) could result in high localized concentrations of reactive oxygen species. Histidine residues at the N-terminus appear to be especially susceptible to site-directed metal ion-catalyzed oxidation (122). Metal-catalyzed oxidation of histidine results in the production of 2-oxo-imidazoline (Fig. 5) (123). By-products of the proposed reaction include aspartic acid. In the metal ion–catalyzed oxidation of polyhistidine, the production of aspartic acid is accompanied by scission of the histidyl peptide bond (120), but it remains unclear whether the scission is a part of the reaction mechanism or merely reflects the instability of the 2-oxo-imidazoline ring to the conditions of isolation and analysis (120,124). Chain scission is not frequently observed in proteins upon histidine oxidation.

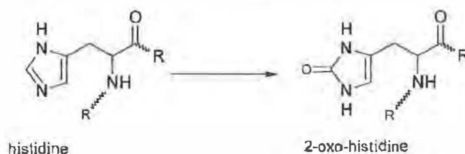

Figure 5 The first product of histidine oxidation, 2-oxo-histidine.

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Cysteine

Metal ion-catalyzed oxidation of cysteine residues usually results in the formation of both intra- and intermolecular disulfide bonds (125,126). Further oxidation of the disulfide results in sulfenic acid.

The mechanism may be summarized as follows (107):

Formation of thiyl radical:

$$RS^- + M^{+n} \Rightarrow RS^\cdot + M^{+(n-1)} \quad (7)$$

Formation of disulfide radical anion:

$$RS^- + RS^\cdot \Rightarrow RS^{\cdot -}SR \quad (8)$$

Formation of superoxide:

$$RS^{-\cdot}SR + O_2 \Rightarrow RSSR + O_2^{\cdot -} \quad (9)$$

Generation of peroxide:

$$RSH + O_2^{\cdot -} + H^+ \Rightarrow RS^\cdot + H_2O_2 \quad (10)$$

Regeneration of M^{+n}:

$$M^{+(n-1)} + O_2 \Rightarrow M^{+n} + O_2^{\cdot -} \quad (11)$$

This mechanism can result in the production of reactive oxygen species capable of further oxidative damage to the disulfide as well as to other residues in the vicinity. When metal ions are made unavailable by chelation with ethylenediaminetetraacetic acid (EDTA), cysteine oxidation is greatly reduced (127). In general, a pH of 6 appears to be optimal for the oxidation of cysteine in proteins (128). At low pH, the protonation of the sulfhydryl (pK_a 8.5) inhibits reaction with the metal ion (Eq. 7). In the alkaline region, electrostatic repulsion of two ionized cysteines is thought to result in an increased separation of the two residues and a reduced reaction rate (Eq. 8). Oxidation in the absence of a nearby thiol has also been observed (129).

Tryptophan

Tryptophan is well known to be a target of reactive oxygen species superoxide (130), singlet oxygen (131), hydroxyl radical (132), and peroxide (133). The most prominent reaction products of tryptophan oxidation appear to be *N'*formylkynurenine and 3-hydroxykynurenine (134). Monohydroxyl derivatives of tryptophan at the 2, 4, 5, 6, and 7 positions have also been observed (Fig. 6). *N'*-formylkynurenine may be also formed by photooxidation (135). Metal ion catalysis of oxidation appears to play a role in the photolytic mechanism (134).

Very little work has been directed toward an understanding of the influence of primary sequence upon tryptophan photooxidation (136,137). It is known that inclusion of Trp in a peptide bond significantly reduces photocatalyzed radical yield (138). At neutral pH and in the presence of dissolved oxygen,

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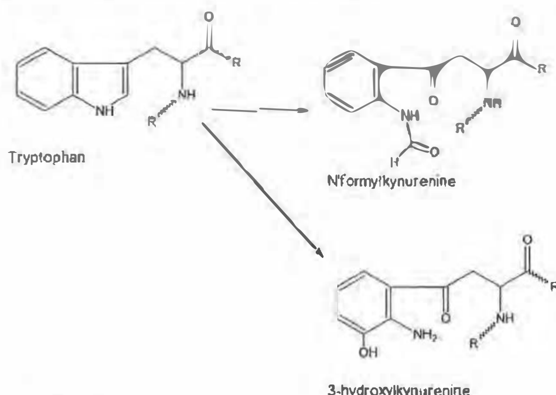

Figure 6 Tryptophan oxidation products.

it has been observed that Gly-Trp photooxidizes at a rate approximately 10-fold that of Trp-Gly. Similarly, Leu-Trp degrades at a rate approximately threefold greater than Trp-Leu. In tripeptides, Gly-Trp-Gly degrades more rapidly than Leu-Trp-Leu. The mechanistic basis of these observations is not clear (136), and additional work remains to be done (137). Under anaerobic conditions, the photolytic degradation rate of tryptophan in peptides is also observed, but it is slowed considerably from the rates observed in the presence of oxygen. In addition, Leu-Trp-Leu exhibits even greater stability over Gly-Trp-Gly. These data indicate that both in the presence and in the absence of oxygen, leucine (with its large side-chain) occupying the C-terminal position next to Trp tends to decrease the degradation rate. Although these data do not provide a sufficient base for a generalized rule, it can be speculated that steric effects have an influence on the rate of photodegradation (137).

Photooxidation of Trp in proteins is known to be directly dependent on the accessibility of the residue to oxygen and solvent water (139,140). Trp residues buried in the core of the protein are less rapidly oxidized than those located at the surface of the molecule (140). Micellar solubilization of hydrophobic peptides appears to protect only the Trp residues located in the core of the micelle (139).

Phenylalanine and Tyrosine

In the presence of copper ion, phenylalanine is oxidized to 2-, 3-, or 4- (tyrosine) hydroxyphenylalanine (141), as shown in Figure 7. Tyrosine may photo- or radio-oxidize to 3,4-dihydrophenylalanine (142), or cross-link with another tyrosine to

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form diotyrosine (143). The latter product may be protease resistant and stable to acid hydrolysis (143). Intermolecular cross-linking would result in increased molecular weight of the reaction product.

Proline

Hydroxyl radical oxidation by the hydroxyl radical of proline (144,145), as well as glutamic acid and aspartic acid (146), is characterized by site-specific cleavage of the polypeptide chain on the C-terminal end of the residue.

Formulation Factors and Oxidation

Overview of Excipient Effects

Shown in Table 2 is a list of first-order rate constants for the reaction of OH with selected formulation excipients that have proved useful in protein systems (101). As with the amino acids shown in Table 1, the rate constants of the excipients listed in Table 2 vary by up to three orders of magnitude. Naturally, ascorbate appears to be the most sensitive to the presence of OH, but so are the proteins (albumin and gelatin) and unsaturated long-chain carboxylate (linoleate). The sensitivity of the latter compound may give rise to concern about the reaction of OH with surfactants containing unsaturated carbon chains as well as lipid-based systems containing triglycerides. A relatively simple iodometric assay suitable for the determination of peroxide levels in surfactants has been published (147).

Iron-catalyzed oxidation has been shown to be rather sensitive to pH (148,149). Phosphate buffer appears to accelerate the reaction as compared to HEPES, but it is possible that trace-metal contamination of the excipients may

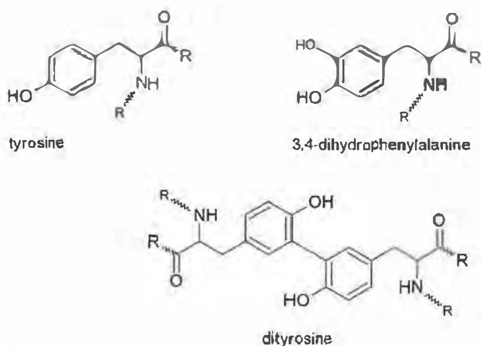

Figure 7 Tyrosine oxidation products.

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Table 2 Rate Constants Reported for the Reaction of $OH^{\bullet}$ with Selected Formulation Excipients

Excipient	pH	*k* (L/mole-s)
Ascorbate	7	1.3×10^{10}
Tartarate	7	6.8×10^{8}
EDTA	4	4×10^{8}
EDTA	9	2×10^{9}
Citric acid	1	5×10^{7}
Glucose	6.5	2.3×10^{9}
Glycerol	7	1.5×10^{9}
Linoleate	7	1.1×10^{10}
Sucrose	7	2.3×10^{9}
Albumin	7	7.8×10^{10}
Carboxymethylcellulose	NL	2×10^{8}
κ-Carrageenan	NL	2.2×10^{8}
Chondroitin-6-SO_4	NL	6.8×10^{8}
Dextran	7	1×10^{8}
Gelatin	NL	9.1×10^{10}
Polyethylene oxide	NL	3×10^{8}
Polyvinylpyrrolidone	7	5×10^{7}

Abbreviations: EDTA, ethylenediaminetetraacetic acid; NL, not listed.
Source: From Ref. 101.

be complicating the observations. In addition, pH effects on metal ion chelation, such as by EDTA, must also be taken into account.

Some of the methods proposed for addressing oxidative damage in a biological matrix may prove useful in the study of proteins and peptides in complex lipid-based drug delivery systems (150). Although lacking specificity, carbonyl assays are often employed as a convenient and rapid measure of the extent of protein damage by reactive oxygen species (151).

Polyethers

Many formulations take advantage of the ability of polyethylene glycol (PEG) and PEG-linked surfactants to stabilize proteins against aggregation and thermal denaturation. The potential ability of these adjuvants to promote oxidation becomes an important consideration. PEG and nonionic polyether surfactants are known to produce peroxides upon aging (152). These peroxides are responsible for drug degradation (153,153) in polyether-containing systems. The oxidation of one such polyether surfactant, polysorbate 80, has been shown to release formaldehyde, a potent protein cross-linking agent (154). Careful purification of PEG-containing adjuvants prior to formulation should minimize this potential degradation mechanism.

Sugars and Polyols

Sugars are often employed as lyoprotectants and as part of the vehicle in the formulation and administration of drugs. Literature reports indicate that moderately

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high concentrations of sugars and polyols seem to inhibit the oxidation of proteins, possibly by serving as hydroxyl radical scavengers (155–157). Although the rate constants for hydroxyl radical reactions with sugars and polyols are not as large as that of ascorbate (Table 2), the high concentrations of the carbohydrate and polymer excipients typically employed in protein/peptide formulations may be responsible for the protective effect.

It was reported in 1996 that various pharmaceutically acceptable sugars and polyols (glycerin, mannitol, glucose, and dextran) were successful in inhibiting ascorbate-Cu(II)–induced oxidation of the protein relaxin and of model peptides Gly-Met-Gly and Gly-His-Gly (158). Results of experiments with glycerin show that contrary to expectations, the protective effect is not the result of radical scavenging. Rather, these authors concluded that the protective effect of the sugars and polyols was due to complexation of transition metal ions. This is in accord with reports indicating weak, but stable, complexes of metal ions with sugars (159,160). Production of reactive oxygen species can be diminished or eliminated by means of competition with the peptide for binding of the metal ion (Eq. 7). Dextran also inhibited oxidation, but a detailed mechanism of the protective effect was not given. The safety and availability of these inexpensive additives make them very attractive as protective agents against oxidation.

The inclusion of glucose in a protein formulation is not without potential risks. Glucose has been shown to participate in oxidation reactions catalyzed by metal ions (161,162). Methionine oxidation products have been observed (163). Upon reaction of glucose with Fe(II), an enediol radical anion intermediate is formed that quickly reacts with molecular oxygen to form the ketoaldehyde (which itself can react with a free amino group on the protein, forming a keto aminomethylol) and the superoxide radical. As a reducing sugar, glucose has been shown to covalently modify relaxin by adding to the side-chains of lysine and arginine and by catalyzing the hydrolysis of the C-terminal serine amide bond. Neither nonreducing sugar (trehalose) nor polyhydric alcohol (mannitol) participates in these reactions (158).

Antioxidants

Antioxidants are commonly employed to protect both small-molecule and peptide/protein drugs from oxidation in pharmaceutical formulations. These are essentially sacrificial targets that have a great tendency to oxidize, consuming pro-oxidant species. The choice of an antioxidant is complicated in proteins and peptides because of the interaction chemistries possible between the antioxidant and the different amino acid side-chains (164). Even antioxidants that are themselves benign to proteins can become potent pro-oxidants in the presence of trace amounts of transition metal ions (e.g., ascorbic acid). In the absence of metal ions, cysteine, as a free amino acid, may act as an effective antioxidant (126,156). By virtue of its singlet oxygen scavenger activity, α-octocopherol has shown protective effects against photooxidation of proteins within lipid membranes (165). Whether this additive is effective in reducing oxidation of proteins or peptides in lipid-based delivery systems, such as liposomes or emulsions, remains unknown.

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Processing and Packaging

Removal of oxygen from solution by degassing processes may be an effective means of inhibiting oxidation in protein and peptide solutions (164,166). Even very low concentrations of oxygen in the headspace will promote oxidation (145). To minimize foaming in protein solutions during degassing, Fransson et al. have suggested cyclic treatments of low temperature and low pressure, followed by exposure to atmospheric-pressure nitrogen gas (83). Packaging in a light-resistant container may be helpful in reducing light-catalyzed oxidation. It should be kept in mind that glass may release minute quantities of metal ions sufficient for metal ion–catalyzed oxidation (167).

Lyophilization

The influence of moisture content on oxidation and other protein-degradation reactions has been explored by a number of authors (4,168,169). Most often, residual moisture enhances the degradation of proteins (4). Hageman has listed both oxidation promotion and oxidation inhibition mechanisms of water (169). The pro-oxidant activities of moisture are believed to include mobilization of catalysts, exposing new reaction sites by swelling, and decreasing viscosity of the sorbed phase. The oxidation-promotion activities of water are thought to be initiated at or near monolayer coverage, where conformational flexibility of the protein is enhanced (168). The oxidation-inhibition activity of sorbed water is thought to arise from retardation of oxygen diffusion, promotion of radical recombination, decreased catalytic effectiveness of transition metals, and dilution of catalyst (169). Much higher water content is required for the modest oxidation-inhibitory effects to become manifest (169). The existence of both pro- and antioxidant effects of moisture would be consistent with the widely varying experimental results observed. For example, Fransson et al. (83) have observed no dependence on moisture of the second-order rate constants for methionine oxidation in insulin-like growth factor, while Pikal et al. (166) found a strong dependence on moisture for methionine oxidation in human growth factor. Luo and Anderson (170) have shown that the mechanism of oxidation of cysteine by hydrogen peroxide in a PVP matrix is significantly altered from that observed in solution. In particular, mobility effects on the cysteine, but not hydrogen peroxide, may be responsible for the observation that cystine is not the only reaction product in the solid state. The results of kinetic studies are consistent with a clustering of water in regions around the polymer and may be a determining factor for the reaction in the solid state.

Clear-cut mechanistic interpretation of these differing oxidation results is not yet possible because of the possible masking effects of formulation additives and moisture-dependent protein conformational states. Residual moisture values in lyophilized proteins of less than 1% tend to be associated with enhanced stability of the protein upon storage (168). In production lots, removal of water to attain such low levels of residual moisture is quite expensive.

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Proteins and Peptides in Polymers

Care must be exercised when attempting to encapsulate pharmaceutical proteins or peptides within a polymer matrix. Cross-linking of methylated dextrans by addition of potassium peroxyodisulfate resulted in significant oxidation of methionine residues in interleukin-2 (171). Addition of tetramethylethylenediamine minimized, but did not eliminate, the oxidation of sensitive amino acids. Similar approaches may be necessary to minimize oxidation of proteins or peptides in other polymer-based systems (76).

ACYLATION IN THE SOLID STATE

Considerable efforts in biomedical research have been devoted to an understanding of posttranslational modification of proteins in biological systems. Other efforts have been geared to the chemical modification of food proteins as a means of controlling processing properties. In pharmaceutical dosage forms, our interest is in preventing modifications of the active ingredients by formulation excipients, such as polymers One of the most frequently employed polymers for this purpose is PLGA, a Food and Drug Administration-approved biocompatible material that releases the incorporated active ingredient by erosion. It has been established that drug substances incorporated into PLGA may be exposed to a highly acidic microenvironment that arises from the hydrolysis of ester bonds of the polymer. Microenvironment pH values as low as 2 have been reported (172). Acid-catalyzed denaturation and chain scission have been attributed to this low pH value (173). More recently, acylation of proteins by polymer components has been observed. Insulin (174), calcitonin (175), octreotide (176), and parathyroid hormone (175) all exhibited evidence of acylation to some extent when encapsulated in PLGA.

Houchin et al. (49) have proposed a mechanism of acylation of peptides by PLGA (Fig. 8). The backbone terminal amino group or the ε-amino of lysine is believed to carry out a nucleophilic attack on the polymer. Water potentiates the reaction, probably by enhancing molecular mobility as well as by promoting hydrolysis of polymer chains. It has been suggested that significant mass loss from the polymer is necessary before acylation can be observed (176). Other studies suggest acylation begins well before significant polymer mass loss (174). Results of model studies of peptide in lactic acid solution do suggest that, in addition to primary amines, other amino acids such as tyrosine and serine may also be sites of acylation (177). Attempts to prevent acylation by PEGylation either of the polymer (178) or of the peptide have been reported. In both cases, there appears to be some reduction in acylation compared to the peptide in the absence of PEGylation, but complete protection was not afforded. In addition, PEGylation of the peptide may result in loss of biological activity. Much additional work remains to be done before a clear strategy can be formulated to prevent acylation by PLGA.

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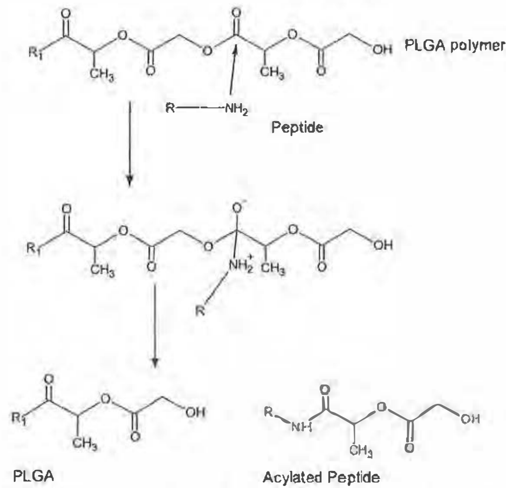

Figure 8 Acylation of peptide by PLGA polymer. *Abbreviation*: PLGA, polylactic-glycolic acid.

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