1

Overview of Protein Formulation and Delivery

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I. PROTEIN AND PEPTIDE REACTIONS

The experience of developing a number of protein and peptide formulations has taught me to look for three principal degradation pathways in formulations of proteins: deamidation, oxidation, and aggregation. In Chap. 2, Bummer and Koppenol cover these three pathways in depth. The problems associated with these protein degradation processes are extensively discussed in this monograph for good reason: they are the constant unwanted companions of development scientists in the protein delivery field.

The deamidation reaction is primarily the result of the hydrolysis of asparagine to aspartic acid. Thus, the reaction is most likely to proceed in an aqueous environment. If the protein is formulated in the solid state, the reaction rate is minimized. As discussed in Chap. 2, this reaction has been extensively studied, and the effects of pH, temperature, and buffers are well known and well documented. This extensive literature gives development scientists the opportunity to systematically optimize solution stability with respect to deamidation. In addition, the reactivity of asparagine is largely determined by neighboring residues, and thus the reactivity of the protein or peptide toward deamidation can be predicted.

Oxidation is in many ways a more complex process than deamidation in that the reactants and catalysts of the reaction are numerous and complex. There are several matters of significant concern; these include the ability to

cause oxidation of specific residues through metal ion catalysis, by vapor phase hydrogen peroxide, photochemically, or through organic solvent, or even to diminish long-term stability in the presence of pharmaceutical excipients. Thus, each unit operation in the production and storage of a protein product is a potential source of oxidation.

The conformational stability of the protein is of fundamental concern in formulating proteins. Problems with conformational stability are often expressed as soluble aggregates and/or insoluble particulates (large aggregates). Aggregated proteins are a significant problem in that they are associated with decreased bioactivity and increased immunogenicity. The potential to produce aggregated forms is often enhanced by exposure of the protein to shear, liquid–air, liquid–solid, and even liquid–liquid interfaces. This means that there is potential to denature and aggregate a protein in almost any unit operation in the downstream processing of a protein, including the formulation of it. However, a protein can often be stabilized against aggregation by optimizing pH, temperature, and ionic strength, and through the use of surfactants.

II. PREFORMULATION AND ANALYTICAL DEVELOPMENT

The development of stability-indicating analytical methods for the drug product usually starts with the preformulation studies. During the preformulation studies, methods are selected and the first evaluation of the protein on short-term stability is conducted. Since one of the most significant purposes of the pre-formulation work is to define the processes that destroy protein integrity and activity, it is critical that the analytical methods chosen for the task indeed be stability indicating. The three basic protein degradation processes (deamidation, oxidation, and aggregation) must be followed by accurate, precise, and simple analytical methods.

Chapters 3 and 4 review analytical test methodology and studies preformulation for proteins and peptides. Deamidation of a protein or a peptide is a common and important chemical decomposition reaction. The deamidation of a protein can be followed by two simple techniques: isoelectric focusing and ion exchange chromatography. Each of these analytical techniques allows the development scientist to follow the disappearance of the starting isoforms and the generation of reaction products. Often, these reaction products (the more deamidated state of the molecule) are easy to isolate and test in bioactivity assays and in pharmacokinetic studies. These activity studies are important steps to take when there is concern that the molecule is prone to deamidation, since the practical consequences of deamidation can be judged scientifically.

Oxidation is often determined using reversed-phase HPLC or by peptide analysis. Aggregation is usually determined by size exclusion chromatography (SEC) or by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). These two techniques give different answers because they address different questions. SDS-PAGE detects and quantifies irreversible aggregates, while SEC will detect noncovalent aggregates as well. The distinction is critical, since the stability of the dosage form may be judged by either or both methods.

During the preformulation and analytical development phase, there is opportunity to determine the correlation between the different analytical techniques and the activity assay. There will be an activity assay that judges the suitability and stability of the dosage form developed. It is imperative to know early in the development process which analytical techniques predict and which degradation processes correlate with losses in activity.

III. SOLUTION FORMULATION STABILITY

In Chap. 5, McGoff and Scher review the issues of protein solubility, aggregation, and adsorption. The authors provide a systematic framework for evaluating the physical stability of proteins. The problems associated with physical instability are serious in that they lead to aggregation, adsorption to surfaces, and loss of biological activity. Thus, formulation studies to determine optimal stabilization conditions should include variables such as temperature, solution pH, buffer ion, salt concentration, protein concentration, and the effect of surfactants. In addition, the protein should be characterized and stabilized against adsorption and/or denaturation at interfaces.

The effect of concentration of the protein on stability of the formulation is also a critical concern. Proteins undergo concentration-dependent aggregation and adsorption, and thus the effect of protein concentration on physical stability must be accounted for. The testing of the adsorption potential of a protein with the range of materials that might be used in packaging or processing is an important part of the formulation effort.

Freeze/thaw stability is also a critical issue for both the formulation and the storage of bulk protein. The development of a liquid formulation for aerosol delivery requires the consideration of additional routes of degradation of the formulated protein. In Chap. 7 on pulmonary delivery, Clark and Shire point out that generating respirable droplets for pulmonary delivery also exposes the protein to the air–water interface and to shear. Thus the formulation scientist must work with methods to reduce this exposure through the use of surfactants.

IV. SOLID STATE FORMULATION STABILITY

The pharmaceutical solid state product of a protein or peptide is usually produced by freeze-drying. A freeze-dried product is produced by a process that, if done correctly, is friendly to protein structure and activity and can prolong the shelf life of the product. The degradation processes such as deamidation, oxidation, aggregation, and hydrolysis are all significantly reduced in a dried solid state protein product. The reason that proteins have enhanced stability in the solid state is that molecular mobility is drastically reduced.

A protein is usually present as an amorphous solid in a lyophilized product, with a glass transition temperature associated with the material. In very rough terms, at temperatures above the glass transition temperature there is significant molecular mobility, while below the glass transition temperature, reduced mobility and reaction rates are achieved. Thus, the stabilization of a protein is dependent on the use of a freeze-drying process and formulation that enhances protein conformation and reduces molecular mobility. Reduced mobility and enhanced protein stability is achieved through temperature, moisture, and excipient control during and after the freeze-drying process. The effect and optimal selection of process conditions and formulation components on protein stabilization are outlined in Chap. 6 on freeze-drying. Chapters 7 and 8 discuss the effect of solid state protein stabilization on pulmonary and controlled delivery.

V. PROTEIN DELIVERY

Some very interesting and challenging problems are associated with drug delivery of proteins. One of the first issues to be faced is the stability of the protein in the body, inasmuch as most drug delivery systems for proteins are implanted or injected by the intramuscular or subcutaneous routes. The body provides an ideal environment for the degradation of a protein, and thus the stability of the product after administration is a critical concern. The 37°C temperature and neutral pH conditions of the human body encourage deamidation reactions. In addition, the higher temperature coupled with moisture will promote molecular mobility and thus accelerate degradation reactions. In addition, the formulation and delivery system must be designed to minimize tissue damage and irritation, since inflammation can also provide routes of protein and peptide degradation. Thus, the formulation of proteins and peptides must also account for response and reaction in biological tissues.

2

Chemical and Physical Considerations in Protein and Peptide Stability

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I. DEAMIDATION

A. 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 asparagine deamidation, since glutamine is significantly less reactive.

B. REACTION MECHANISM

The primary reaction mechanism for the deamidation of Asn in water-accessible regions of peptides and proteins at neutral pH is shown in Fig. 1. For the present, discussion is confined to the intramolecular mechanism, uncomplicated by adjacent amino acids at other points in the primary sequence. The key step is the formation of a deprotonated amide nitrogen, which carries out the rate-determining nucleophilic attack on the side chain carbonyl, resulting in the formation of the five-membered ring succinimide intermediate. For such

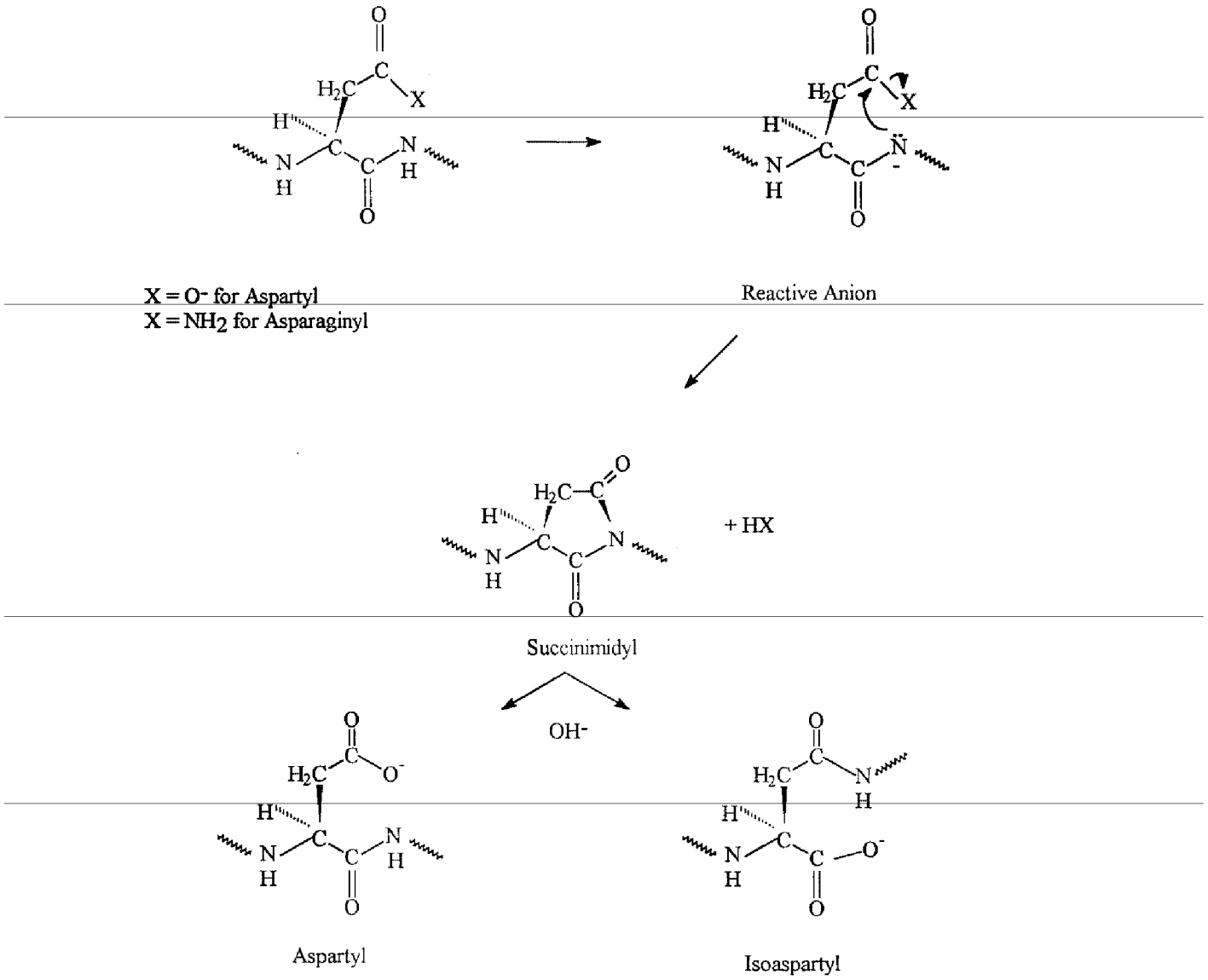

Fig. 1 Proposed reaction mechanism for deamidation. Note the formation of the succinimidyl intermediate.

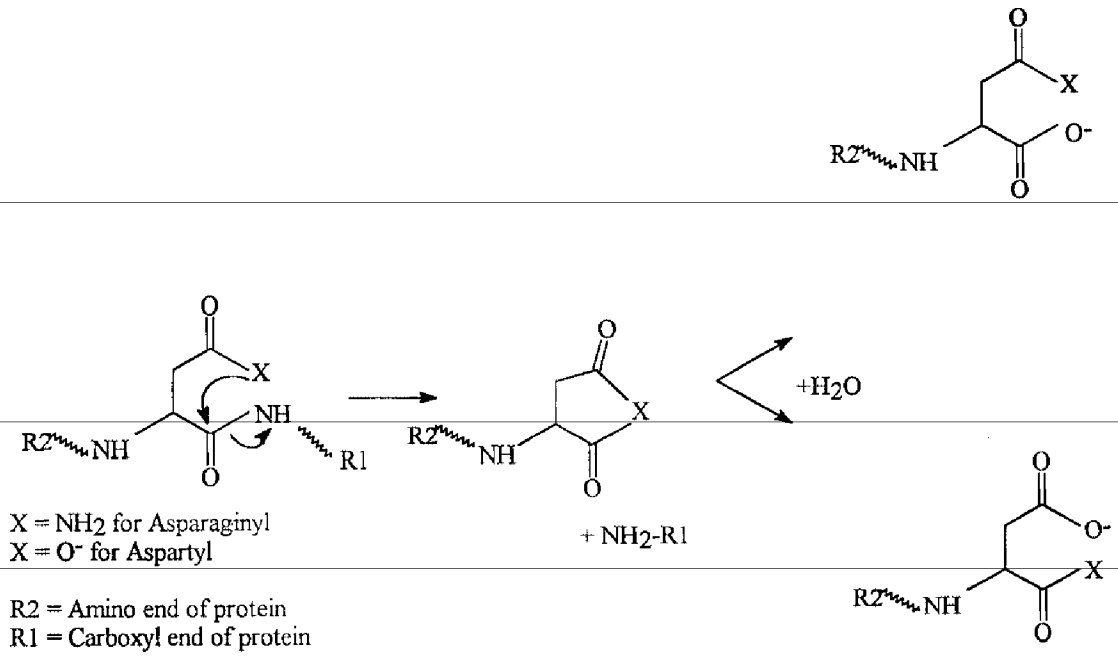

Fig. 2 Proposed reaction mechanism for main chain cleavage by aspartyl or asparaginyl residues.

a reaction, the leaving group must be easily protonated, and in this case it is responsible for the 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). The reaction also appears to be sensitive to racemization at the α carbon, resulting in mixtures of D- and L-isomers (10,13,14). No matter the final product, the rate of degradation of the parent peptide in aqueous media most often follows pseudo-first-order kinetics (15,16).

A number of other alternative reactions are possible. The most prevelant appears to be a nucleophilic attack of the Asn side chain amide nitrogen on the peptide carbonyl, resulting in main chain cleavage (10,15,17). This reaction (see 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.

C. 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

(15,18). Under these extreme conditions, the reaction is often complicated by main chain cleavage. Deamidation by this mechanism is not likely to produce isoaspartate or significant racemization (15).

In the cyclic imide mechanism present under more moderate conditions, the effect of pH is the result of two opposing reactions: (a) deprotonation of the peptide bond nitrogen promoting the reaction and (b) protonation of the side chain leaving group, inhibiting the reaction. In deamidation reactions of short chain peptides uncomplicated by structural alterations or covalent dimerization (19), the pH–rate profiles exhibit the expected ''V'' shape with a minimum occurring in the pH range of 3–4 (15). 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 (15,18). The pH minimum in the deamidation reaction measured in vitro for proteins may (20) or may not (21) fall in the same range as that of simple peptides. Overall pH-dependent effects may be modified by additional, structure-dependent factors, such as dihedral angle flexibility, water accessibility, and proximity of neighboring amino acid side chains (see below).

D. Effect of Temperature

The temperature dependence of deamidation rate has been studied in a variety of simple peptides in solution (15,22,23). 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 (neutral to alkaline pH).

The deamidation rate of proteins also shows temperature dependence (21,24,25) under neutral pH. The inactivation of proteins as a function of temperature is much more difficult to isolate solely to deamination for several reasons: competing reactions at other side chains, thermal instability of the structure of the protein, or main chain hydrolysis. For deamination reactions alone, rate acceleration in protein may be due to enhanced flexibility in the protein, allowing more rapid formation of the cyclic imide formation (26), 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 deamination rate constant was observed to increase about an order of magnitude between 40 and 70°C (27). 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 5. The authors related this temperature difference to the difference in mechanism that may occur as a function of pH. In direct hydrolysis at low pH, water is a necessary reactant. In the cyclic imide mechanism at neutral to alkaline pH, deamidation can occur without the availability of water.

E. 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 may be employed in protein isolation and purification procedures (28). Important clues to stabilization strategies can be gained from these studies. In all 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).

1. Buffers

Buffer catalysis appears to occur in some but not all peptides and proteins (5). Bicarbonate (15) and glycine (12) buffers appear to accelerate deamidation. On one hand, phosphate ion has been shown to catalyze deamidation, both in peptides and proteins (12,13,15,29–31), generally in the concentration range of 0–20 mM. On the other hand, Lura and Schrich (32) 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 phosphate ion catalyzes deamidation was challenged in 1995 by Tomizawa et al. (13), who found that the deamidation rate of lysozyme at 100°C did not exhibit the expected linear relationship of deamidation rate on phosphate concentration. Brennan and Clarke (16) have suggested that phosphate ion, or other buffer ingredients, could act on the aqueous solvent to increase basicity of water molecules without forming free hydroxide ions. Experimental data directly supporting this speculation have not yet appeared in the literature.

2. 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 activity 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 (20). 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 phosphate ion (13).

In reviewing the data above, Brennan and Clarke (16) tentatively attributed the promotion of deamidation by elevated levels of ions to enhanced stabilization 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.

3. 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 (16). Following this hypothesis, Brennan and Clarke (33) analyzed succinimide formation of the peptide Val-Tyr-Pro-Asn-Gly-Ala [the same peptide employed by Patel and Borchardt (15) in studies of pH effects in aqueous solution] as a function of organic cosolvent (ethanol, glycerol, and dioxane) at constant pH and ionic strength. The lower dielectric constant media resulted in 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 alcohols, 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 do not make for attractive pharmaceutical adjuvants. The effects of low dielectric may still supply a rational for the solubilization of peptides in aqueous surfactant systems, where the hydrophobic region of the micell or a liposome could potentially enhance stabilization of the Asn residues from deamidation.

As pointed out by Brennan and Clarke (16), the results of experiments in organic solvents can have implications on 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 (a value of 78 at 25°C; Ref. 34), 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 more in line with that of ethanol or dioxane (35), reaction rates would be expected to be considerably slower.

F. Peptide and Protein Structure

The ability to identify which Asn or Gln residues in a therapeutic protein or peptide 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.

1. 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 extended to illucidate 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.

a. Effect of Amino Acids Preceding Asn or Gln

In an extended series of early studies, Robinson and coworkers (22) 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 three-fold.
2. In peptides Gly-X-Asn-Ala-Gly, steric hinderance by un-ionized 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 later case, $t_{1/2}$ ranged from 418 to 3278 days, in accordance with the diminished reactivity of glutamine.
3. For the same host peptide, when X side chain was charged, the deamidation rate of Asn followed the rank order of Asp > Glu > Lys > Arg.

b. 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 (36). More recent studies of ACTH-like sequence hexapeptide Val-Tyr-Pro-Asn-Gly-Ala under physiologic conditions (37) has 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 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. 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.

Steric hinderance 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 prolyl amide nitrogen to attack the Asn side chain (10). For other residues, electron-inducing effects of the side chain of the following residue may inhibit deprotonation of the peptide bond nitrogen, inhibiting deamidation (15,38).

In more recent studies, deamidation of Val-Tyr-X-Asn-Y-Ala, a peptide sequence derived from ACTH, the adrenocorticotropic hormone, was examined with different residues in both flanking positions (39). When X is 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 asparagine did seem to accelerate main chain cleavage products.

Some of the general rules for peptides may also show higher levels of dependence on primary sequence. Tyler-Cross and Schirch (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 (22) 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 (15), 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.

2. Secondary and Tertiary Structure

X-ray or 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 (40). It is beyond the intent 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 (see, e.g., Ref. 6). For the most part, detailed mechanisms relating the secondary and tertiary structure of proteins to enhancement of rates of deamidation are not yet available.

Clear differences in deamidation rates of some proteins are evident when native and denatured states are compared (13,41). Denaturation is thought to enhance main chain flexibility and water accessibility (40). 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. Inasmuch as such angles tend to be energetically unfavorable (42) 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.

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 NH of the fourth residue along the chain. The resulting bond is close to the optimal geometry, and therefore maximal energy, for such an interaction (42). 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 (43), Chazin and Kossiakoff (40) 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 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 (40). Perhaps studies modeled along the lines of guest–host relationships would be helpful in elucidating further the influence of secondary structure on deamidation (44).

G. Effects of Deamidation on Structure

In 1994, in an extensive and detailed series of studies, Darrington and Anderson showed that deamidation strongly influences the noncovalent self-association (45) and covalent dimer formation (45,46) of human insulin. The noncovalent dimer formation of triosephosphatase (47) 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 (41). The isoelectric point

of the deamidated molecule is shifted toward lower values, possibly resulting in modified potential for adsorption to solid surfaces (48). Foamability of protein solutions subject to deamidation is greatly enhanced, probably because of partial unfolding (41).

Deamidation can destabilize a protein, making thermal (47) or chemical (13) denaturation more likely. Folding patterns may be influenced (49,50), and changes in secondary structure can result (47). Other proteins appear to be resistant to structure alterations secondary to deamidation (51–56).

II. OXIDATION

A. Introduction

Oxidation has been identified as one of the major degradation pathways in proteins and peptides and can occur during all steps of processing, from protein isolation to purification and storage (57,58). An extensive listing of proteins undergoing oxidation, the primary amino acids involved, and the biological activity of the products has been provided (4). 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 (59,60). There are 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 (57,61).

B. Oxidation in Pharmaceutical Proteins and Peptides

In living systems, a variety of well-characterized reactive oxygens are produced (62,63). In pharmaceutical formulations, identifying a single oxidation initiator is often difficult, since a variety of initiation possibilities exist, such as photochemical (4,64,65), metal-ion-catalyzed (66,67), high energy γ-radiation (66), and organic additives (68,69). Even something as seemingly simple as sonication appears to promote the generation of reactive oxygen species (70,71). 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 (65,72–74).

In pharmaceutical proteins, transition metal ion catalysis of oxidation has received the lion's share of attention (73,75,76), while much less attention has been devoted to light energy and thermal energy (64,65).

1. *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 (66). Since the metal-ion-catalyzed systems tend to be amenable to laboratory manipulations, they have been employed in stability studies (73,75,76). More importantly, trace levels of metal ions known to initiate oxidation are often present as contaminants in pharmaceutical systems (57), 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 Eq. 1.

$$Fe(II) + O_2 \Leftrightarrow Fe(III) + O_2^{\bullet -} \quad (1)$$

The superoxide radical is not stable at neutral pH and undergoes dismutation to form hydrogen peroxide by Eq. 2.

$$2OO_2^{\bullet -} + 2H^+ \Rightarrow H_2O_2 + O_2 \quad (2)$$

Hydrogen peroxide reacts further to produce hydroxyl radicals (OH·) by Eq. 3.

$$H_2O_2 + Fe(II) \Rightarrow Fe(III) + OH^- + OH\cdot \quad (3)$$

Hydroxyl radicals are capable of abstracting hydrogen atoms with bond energies less than 89 kcal/mol (77–79) producing organic radicals by Eq. 4.

$$OH\cdot + RH \Rightarrow H_2O + ROO\cdot \quad (4)$$

The organic radical (ROO·) is capable of entering into a whole variety of chain reaction propagation and termination reactions (75). Overall, Eqs. 1–4 show the production of four different reactive oxygen species, each able to oxidize pharmaceutical proteins. In solutions of free amino acids, oxidation by OH· shows a strong dependence on bicarbonate ion (66,80). 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. Bicarbonate appears not to be a necessary reactant of protein or peptide oxidation.

2. 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 (Esq. 1–4), 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 (75,76). 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 (76):

$$D\text{–}Fe(II) + O_2 + H^+ \Rightarrow D\text{–}Fe(III)\text{–}OOH \quad (5)$$

where 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 effective formulation protective agents (76). The terminal hydroxyl group of serine, the free carboxyl group of aspartic and glutamic acids, the imidazole ring of histidine, and the free amino or free carboxyl groups of N-terminal or C-terminal (respectively) residues have all been suggested to participate in binding metal ions to proteins (80). 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.

3. Oxidation by Hydrogen Peroxide Addition

Addition of hydrogen peroxide has been employed as a means to study oxidation of proteins (72,81,82), 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 (83) while in other instances, oxidation of cysteine and tryptophan residues also occurs (84). Hydrogen peroxide 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 (83). It has been proposed that *t*-butyl hydroperoxide may be a highly specific oxidizer of surface-localized methionine residues (83).

C. Specific Amino Acid Side Chains

1. Methionine

Methionine has been identified as one of the most easily oxidized amino acids in proteins, 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 (85). The reaction product of methionine oxidation is the corresponding sulfoxide and, under more strenuous oxidation conditions, the sulfone (Fig. 3). 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 (65). Peroxide (86), peroxyl radicals (87), singlet oxygen (86), and hydroxyl radical (75) 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 controversy (88, 89).

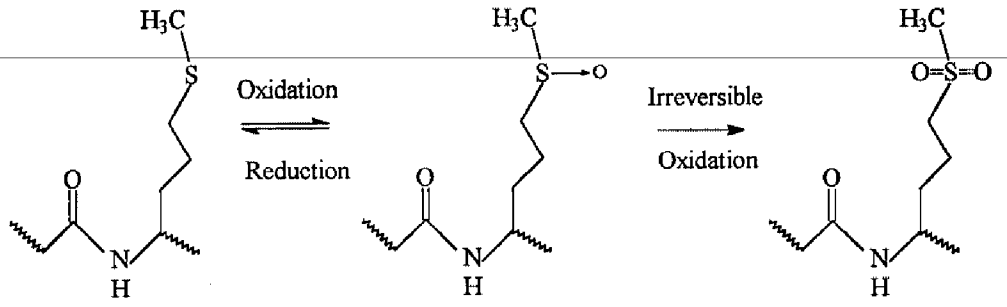

Fig. 3 Oxidation of methionine first to the sulfoxide and then to sulfone derivatives.

The reaction mechanisms for proteins in pharmaceutical systems are incomplete because not all products and intermediates are known. An excellent example of oxidation induced by a variety of pro-oxidants in a single recombinant protein and the experimental difficulties encountered is presented next.

a. Oxidation of Methionine in Recombinant Human Relaxin

Photocatalyzed oxidation. A series of papers spanning the 1990s studied methionine oxidation initiated by light (65), hydrogen peroxide (72), and ascorbic acid–Cu(II) (73) in recombinant human relaxin. Upon exposure to light of an intensity of 3600 candles for 5–17 days, both methionine residues, Met-B4 and Met-B25, located on the surface region of the B-chain, were oxidized to the sulfoxide derivative (65). 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.

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 (72). Three products were isolated, the monosulfoxide at either 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 more rapid than that 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 (73). Approximately 80% of protein was lost from solution within 25 minutes at pH 7–8. Chromatographic results indicated that the aggregate formed was not held together by covalent forces. In a second significant contrast to the study above (72), in the presence of ascorbic acid–Cu(II), investigators observed oxidation of histadine, methionine, and lysine (73). One final important difference is that Met-B4 was oxidized preferentally 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 methio-

nine, or any other residue, is of primary importance in setting down complete reaction mechanisms.

b. 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. Trailblazing work of Li, Schoneich, and Borchardt, and their colleagues (75,76,90) has begun to address 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 peptides. Considerable efforts have been expended with specific radical scavengers to identify the reactive oxygen species responsible for oxidation.

c. Buffers and pH

Using the pro-oxidant systems of DTT/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 of all peptides followed first-order kinetics, while mass balance comparisons showed that sulfoxide was not the terminal degradation product. The rate of loss of parent peptide did not vary with pH in the range 6–8.1. The rate of loss was observed to accelerate with pH beyond this range.

Li et al. (90) found 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 (90). Buffer species such as Tris or HEPES have a weak affinity for metal ions (91) 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 (92), 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