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MINIREVIEW

Solid-State Chemical Stability of Proteins and Peptides

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Abstract ☐ Peptide and protein drugs are often formulated in the solid-state to provide stabilization during storage. However, reactions can occur in the solid-state, leading to degradation and inactivation of these agents. This review summarizes the major chemical reactions affecting proteins and peptides in the solid-state: deamidation, peptide bond cleavage, oxidation, the Maillard reaction, β-elimination, and dimerization/aggregation. Physical and chemical factors influencing these reactions are also discussed. These include temperature, moisture content, excipients, and the physical state of the formulation (amorphous vs crystalline). The review is intended to serve as an aid for those involved in formulation, and to stimulate further research on the determinants of peptide and protein reactivity in the solid-state.

Introduction

In the last two decades, proteins and peptides have become an important class of potent therapeutic drugs. However, their susceptibility to chemical degradation in solution presents a challenge in the development of stable protein pharmaceuticals.[1] As a result, many polypeptide drugs are formulated as lyophilized or freeze-dried products to prolong their shelf life.[2–4] While a "dry" formulation is generally more stable than the corresponding aqueous formulation, chemical degradation reactions can still occur.[2–4] In some cases, protein stability in the solid state is less than or comparable to that in solution.[5,6]

Factors that may impact the chemical stability of proteins and peptides in the solid-state include residual moisture and the excipient(s) used in a formulation. Excipients, such as polymers, are often included in protein and peptide formulations to protect the drug from degradation during processing and/or storage or to act as a matrix for controlled release. This review presents an overview of the chemical degradation reactions common to proteins and peptides in the solid state, and of our current knowledge regarding the effects of formulation and storage factors on peptide and protein stability in these systems.

The degradation pathways of proteins in the solid state can be classified into two types: chemical and physical. Chemical instability involves covalent modification of a protein or amino acid residue to produce a new molecule via bond cleavage, bond formation, rearrangement, or substitution. These chemical processes include such reactions as deamidation at asparagine (Asn) and glutamine (Glu) residues,[7] oxidation of sulfur atoms at cysteine (Cys) and methionine (Met) residues, disulfide exchange at Cys, and hydrolysis of aspartate (Asp) and glutamate (Glu) residues.[8] Physical instability refers to changes in the three-dimensional conformational integrity of the protein and does not necessarily involve covalent modification. These physical processes include denaturation, aggregation, precipitation, and adsorption to surfaces.[1] Chemical instabilities, such as deamidation and disulfide bond cleavage, may lead to physical instabilities, and vice versa. The physical instabilities of proteins will not be discussed in detail here, since the focus is chemical instability. The reader may refer to a recent article on the physical instability of proteins for further discussion.[9] Instead, this review will discuss reactions and factors that contribute to the chemical instability of proteins and peptides in the solid-state.

The different types of chemical reactions that contribute

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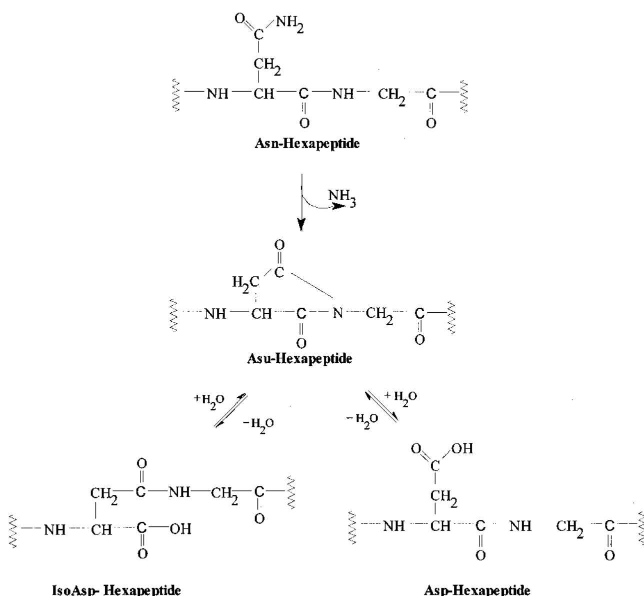


Scheme 1—Deamidation.

to protein and peptide instability in the solid-state are first presented. These include deamidation, peptide bond cleavage, oxidation, the Maillard reaction, β-elimination, and covalent dimerization or aggregation. In the next section, formulation factors that affect chemical stability, such as temperature, moisture, and excipients, are discussed. The review concludes with a summary and a discussion of the implications for future research.

Solid-State Reactions of Peptides and Proteins

Deamidation—Chemical instability in the solid state due to deamidation has been observed for human growth hormone (hGH),[4,10] recombinant human interleukin-1 receptor antagonist,[11] recombinant bovine somatotropin (growth hormone),[12] and insulin.[13,14] While there have been numerous mechanistic studies of protein and peptide deamidation in solution,[7,15–19] few such studies have been reported for deamidation in the solid state. Two studies which provide a mechanistic perspective on deamidation in solids are summarized below.

The stability and mechanism of degradation of the Asn-hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) were studied in solid formulations lyophilized from acidic solutions ranging from pH 3–5. The main degradation pathway for Asn-hexapeptide in the pH 3 formulation is deamidation via hydrolysis of the Asn side chain to produce the Asp-hexapeptide, which is further hydrolyzed at the Asp-Gly amide bond to generate a small quantity of tetrapeptide (Scheme 1).[18] As the pH of the solution prior to lyophilization increases from 3 to 5, intramolecular attack of the carbonyl center of the Asn side chain by the amide nitrogen of the succeeding amino acid to form a cyclic imide intermediate becomes more prominent, as evidenced by an increase in the cyclic imide in the product distribution (Scheme 1).[18] In solution at pH 5, the cyclic imide is hydrolyzed to form the isoAsp-hexapeptide, which is the dominant degradation product;[15] however, the isoAsp-hexapeptide was not observed in the solid state.[18] The absence of the isoAsp-hexapeptide in the product distribution may be due to the low level of water available for hydrolysis in the solid state. The mechanism of deamidation for the Asn-hexapeptide in the solid state was found to be similar to that in solution.[18] In an extension of this work, we have recently investigated the deamidation of this peptide in solid poly(vinyl alcohol) and poly(vinyl pyrrolidone) matrixes.[20–22] As in the lyophilized peptide, the mechanism of deamidation appears to be similar to that in solution, but the kinetics and product distribution are altered, particularly in matrices of low water content.

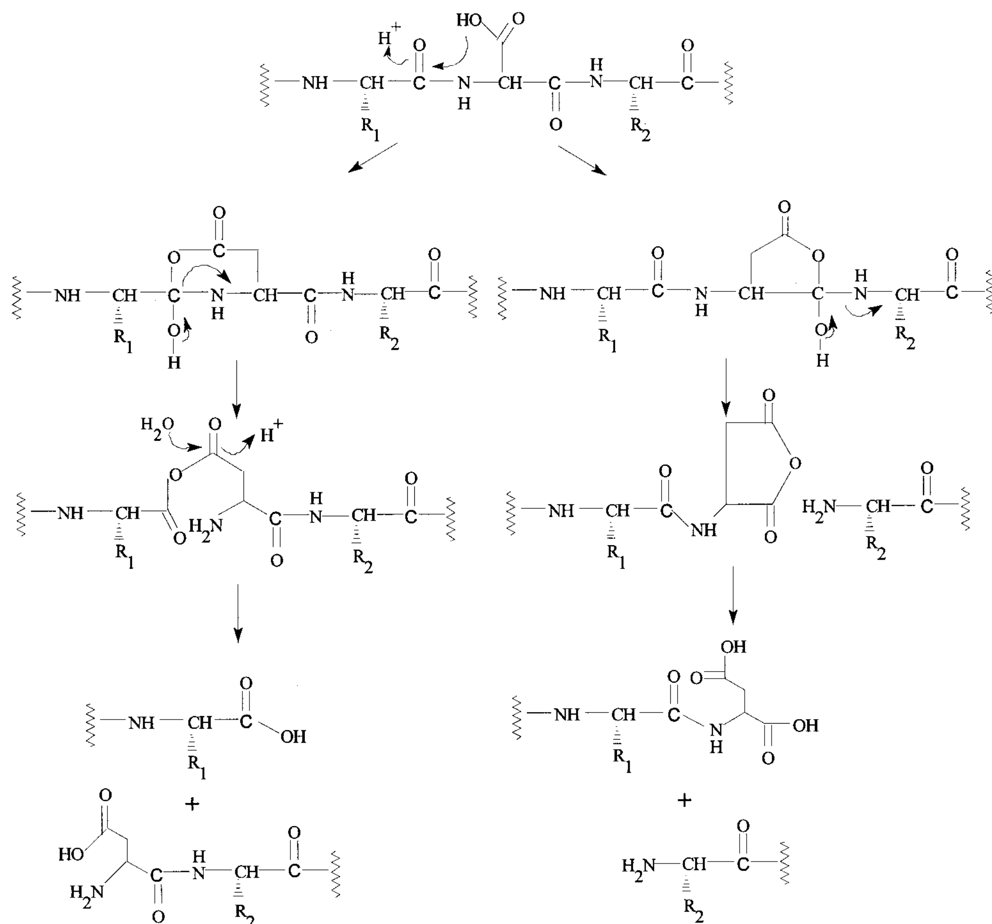
Human insulin has also been observed to undergo deamidation in the solid-state via a mechanism similar to that in solution.[13] In insulin formulations lyophilized from acidic solutions (pH 3–5), the rate-determining first step involves intramolecular nucleophilic attack of the C-terminal Asn_{A21} carboxylic acid onto the side-chain amide carbonyl to release ammonium and to form a reactive cyclic anhydride intermediate, which can further react with various nucleophiles.[13] The cyclic anhydride intermediate may react with water to form [$desamido_{A21}$] insulin,[13] and may also react with another molecule of insulin to form covalent dimers.[13] While the cyclic imide intermediates formed during Asn-hexapeptide deamidation in the solid state were observed to accumulate,[23] the cyclic anhydride intermediate formed during insulin deamidation did not.[13] Strickley and Anderson were able to verify that insulin deamidation proceeds via formation of a cyclic anhydride by using aniline trapping of the intermediate.[13] Consistent with these findings, Pikal and Rigsbee observed that the deamidation of human insulin occurs predominantly at Asn_{A21}, except at high relative humidity when deamidation at Asn_{B3} is more prevalent.[14]

Similar to the degradation of the Asn-hexapeptide discussed previously, the solid-state degradation of a model Asp-hexapeptide (Val-Tyr-Pro-Asp-Gly-Ala) is dependent on the pH of the bulk solution prior to lyophilization. This value is often referred to as the "pH" of the solid, since the true hydrogen ion activity is difficult to measure and pH is technically undefined in the solid-state. Under acidic conditions ("pH" 3.5 and 5.0), the Asp-hexapeptide mainly decomposes to produce a cyclic imide intermediate via base-catalyzed intramolecular cyclization.[23] Hydrolysis of the Asp-Gly amide bond also occurs but to a lesser extent. Under neutral and basic conditions ("pH" 6.5 and 8.0), the Asp-hexapeptide degrades exclusively via intramolecular cyclization to produce the Asu-hexapeptide, which is further hydrolyzed to form the isoAsp-hexapeptide.[23] At "pH" 8, the isoAsp-hexapeptide is the dominant degradation product,[23] similar to that observed in solution.[24]

Peptide Bond Cleavage—A second common degradation pathway for peptides and proteins involves cleavage of the peptide bond. Representative pathways of peptide bond cleavage are shown in Scheme 2. Lyophilized human relaxin formulated with glucose can undergo hydrolytic cleavage of the C-terminal serine (Ser) residue on the B-chain (Trp_{28}-Ser_{29}-COOH) upon storage at 40 °C.[25] This observation was supported by a reduction in molecular mass corresponding to the loss of Ser from fragment T5-T9 of relaxin, as verified by liquid chromatography/mass spectroscopy (LC/MS) and tryptic digest.[25] Li et al. proposed that this cleavage involved an initial reaction of the Ser hydroxyl group with glucose followed by subsequent hydrolysis of the Trp-Ser bond via a cyclic intermediate.[25]

In the solid state, the major degradation pathway of aspartame (α-aspartylphenylalanine methyl ester, APM) is intermolecular cyclization to form exclusively diketopiperazine (DKP) with the elimination of methanol.[26] In solution, the degradation of aspartame at neutral and basic pH also occurs mainly via cyclization to form diketopiperazine (DKP) or hydrolysis at the ester linkage to form α-aspartylphenylalanine (AP) and methanol.[27] Since water was absent in the aspartame solid-state study, no hydrolysis products were observed.[26]

The instability of the undecapeptide substance P (SP) in the solid state also proceeds through diketopiperzine



Scheme 2—Proteolysis.

formation. The main pathway of decomposition consists of the sequential release of N-terminal dipeptides via their diketopiperazines, cyclo(Arg-Pro) and cyclo(Lys-Pro).[28] Under the conditions studied, the release of N-terminal dipeptides dominates over other possible routes of spontaneous modification, such as oxidation and deamidation.[28]

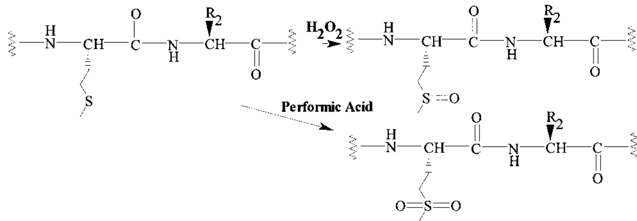
Oxidation—The side chains of His, Met, Cys, Trp, and Tyr residues in proteins are potential sites for oxidation (Scheme 3).[1] A major chemical decomposition pathway for human growth hormone (hGH) in the solid state is methionine oxidation at Met_{14} to form the sulfoxide.[10] Even with minimal oxygen (~0.05%) in the vial headspace, decomposition via oxidation is comparable to or greater than that due to the alternative reaction of deamidation.[4] Storage of lyophilized hGH in an oxygen atmosphere results in greater decomposition than storage in a nitrogen atmosphere.[29] As in solution, atmospheric oxygen can easily oxidize Met residues in the solid state, leading to chemical instability and loss of biological activity.

Human insulin-like growth factor I (hIGF-I), lyophilized from phosphate buffer, also undergoes oxidation at its Met residue.[30] There were no significant differences in reaction rates (second-order kinetics) between solution and solid states.[30] However, Met oxidation in the solid-state constitutes a greater fraction of the total protein modification than in solution.[30] Both oxygen content and light exposure affect the oxidation rate.[30] Exposure to light increases the oxidation rate by a factor of 30.[30] This increase in oxidation rate with exposure to light and oxygen suggests that photooxidation and molecular oxygen may be involved in the generation of radicals. However, no further experiments were conducted to determine the nature of the radicals involved or the mechanism of Met oxidation in the solid state.

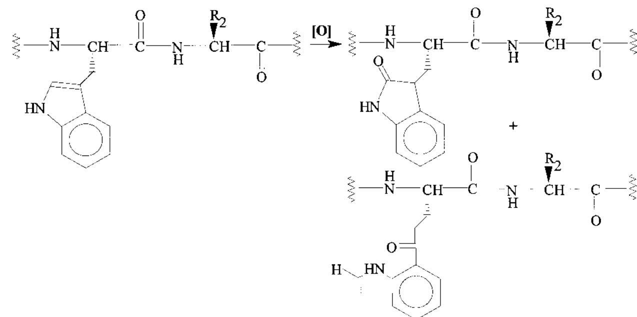
The oxidative deamidation of a cyclic hexapeptide, acetylcysteine-asparagine-(5,5-dimethyl-4-thiazolidinecarbonyl)-(4-(aminomethyl)phenylalanine)-glycine-aspartic acid-cysteine cyclic disulfide, in a lyophilized mannitol formulation does not appear to depend on molecular oxygen as a reactive species.[31] The oxidation reaction occurs at the aminomethyl phenylalanine moiety to form a benzaldehyde derivative.[31] This oxidative degradant is not detected in the neat solid drug stored under atmospheric oxygen, suggesting that oxygen is not involved in the reaction.[31] Instead, the decomposition of the heptapeptide may be due to a reaction with reducing sugar impurities in the mannitol excipient.[31] The proposed mechanism involves (1) formation of a Schiff base from the peptide primary amine reacting with the carbonyl of the aldehydic group on the reducing sugar, (2) tautomerization to a more stable configuration, conjugated with the phenyl group, and (3) hydrolytic cleavage of the new Schiff base to generate the observed aldehyde derivative.[31] The first part of this proposed reaction, the formation of the Schiff base, proceeds via a mechanism similar to the Maillard reaction, discussed below.

Maillard Reaction—The food industry has studied extensively the nonenzymatic browning of food due to the Maillard reaction, which results from reducing sugars reacting with either amino or free amine groups in proteins, leading to changes in both the chemical and physiological properties of the proteins (Scheme 4).[32] The first phase of the Maillard reaction involves a condensation reaction between the carbonyl of a reducing sugar and an amino group to form an N-substituted glycosylamine, which then converts to a Schiff base and a molecule of water.[33] Subsequent cyclization and isomerization (Amadori rearrangement) result in derivatives which cause discoloration

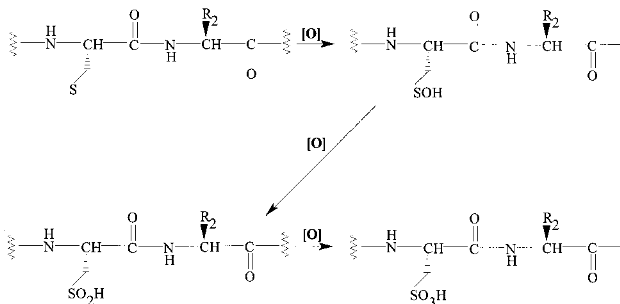
A. Met



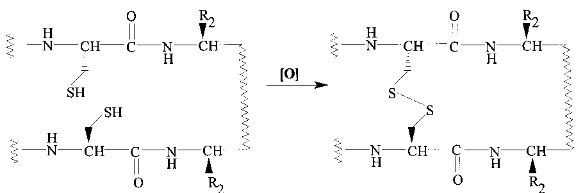
B. Trp



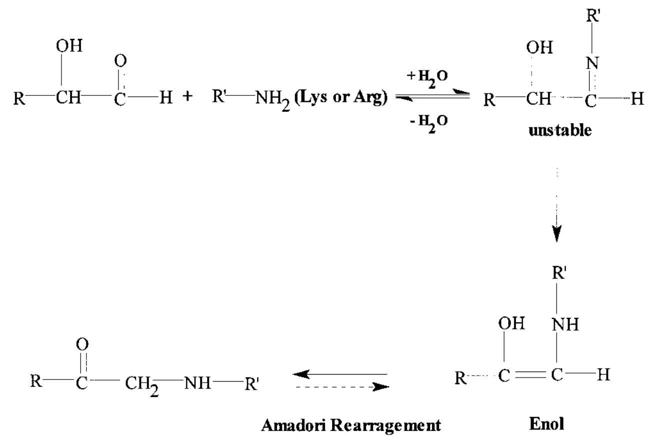
C. Cys



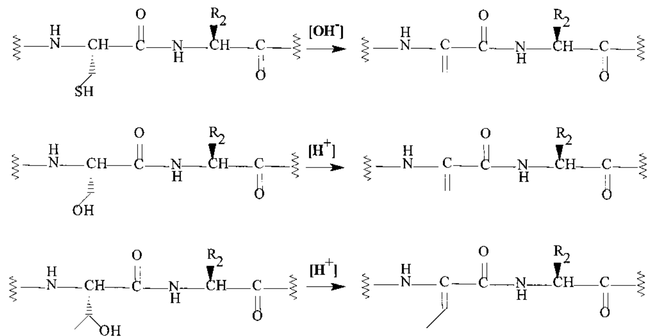
D. Disulfide Bond



Scheme 3—Oxidation.



Scheme 4—Maillard Reaction.



Scheme 5—β Elimination.

(nonenzymatic browning) of the formulation.[33] This type of covalent modification is a problem in the solid state because the initial aminocarbonyl condensation reaction to form the Schiff base is accelerated in the low moisture range.[25,34]

Lyophilized human relaxin (Rlx) formulated with glucose was observed to degrade via the Maillard reaction to form adducts with glucose.[25] The resulting adducts were shown by liquid chromatography/mass spectroscopy (LC/MS) to contain up to four glucose molecules covalently attached to Rlx.[25] With tryptic digestion, the individual reaction sites on Rlx were identified as including Lys_{A9}, Lys_{B9}, and Arg_{B17}.[25] The fourth reaction site was hypothesized to be either Lys_{A17} or Arg_{B13} on the T2-T7 fragment, which showed a broadened peak on the tryptic map, but positive identification was not possible using mass spectroscopy.[25]

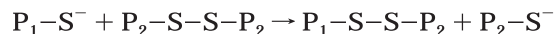
A review of the food literature prior to 1966 by Goldblith and Tannenbaum revealed that lysine loss in foods is primarily due to the Maillard reaction.[35] Lysine is usually lost more rapidly than the other amino acids because of its free ϵ-amino group, which will react easily with the carbonyl of reducing sugars.[33] However, other residues, such as arginine, asparagine, and glutamine, have been observed to react with reducing sugars also.[25,34]

***β*-Elimination**—Lyophilized bovine insulin has been observed to degrade rapidly with increased water content to produce both covalent and noncovalent aggregates after incubation at 50 °C at various relative humidities.[36] Costantino et al. hypothesized that the reducible covalent interactions were due to thiol-catalyzed disulfide exchange.[36] They speculated that these free thiol groups resulted from the β-elimination of intact disulfide bonds in insulin.[36] The proposed mechanism for β-elimination involves hydroxide ion catalyzed cleavage of a carbon–sulfur bond (cysteine) (Scheme 5A), resulting in two new residues, dehydroalanine and thiocysteine.[36] Dehydroalanine can then react with lysine to form a lysinoalanine cross-link, while thiocysteine can undergo further decomposition to form thiol-containing products, such as hydrosulfide ions (free thiols).[36] A 5-fold increase in the level of free thiols was measured in lyophilized insulin after incubation at 50 °C and 96% relative humidity.[36] The free thiols formed during solid state aggregation were predominantly low molecular weight (<3000 Da), perhaps hydrosulfide ions.[36]

Covalent Dimerization and Aggregation—Dimerization and aggregation differ from the other types of degradation reactions discussed in this review in that they are not the result of a single chemical change. In fact, many different types of chemical or physical changes can induce the formation of dimers or aggregates. A brief description of chemically induced dimerization and aggregation is included here because of its importance in the pharmaceutical industry, and because much of the published literature on protein stability uses aggregate formation as

a key stability indicator. Dimerization or aggregation via covalent cross-linkage can be categorized into two types: reducible (via disulfide exchange) and nonreducible.

Reducible Dimerization and Aggregation—Lyophilized bovine serum albumin, ovalbumin, glucose oxidase, and β-lactoglobulin were observed to form covalent intermolecular disulfide linkages via a thiol−disulfide interchange reaction (Scheme 3D).[37,38] Liu et al. postulated that the intermolecular thiol−disulfide interchange results from the ionized thiol on one albumin molecule attacking the disulfide linkage of another albumin molecule:



$$P_1{-}S^- + P_2{-}S{-}S{-}P_2 \rightarrow P_1{-}S{-}S{-}P_2 + P_2{-}S^-$$

where P1 and P2 are the first and second protein molecules.[37] Thiolate ions rather than thiols are the reactive species in this reaction, since a decrease in the initial solution pH resulted in a slower aggregation rate while an increase in pH led to a more rapid reaction.[37] Free thiols are both necessary and sufficient for the moisture-induced aggregation to occur, since S-alkylated BSA (no free sulfhydryl groups) does not undergo aggregation.[37]

Moisture-induced aggregation of bovine insulin occurred via both noncovalent and covalent interactions. The covalent interactions were reportedly due to intermolecular disulfide bonds as evidenced by the dissolution of 21% of the aggregates upon treatment with a reducing agent, 10 mM dithioerythritol, and 1 mM EDTA to prevent its autoxidation.[36] Costantino et al. postulated that β-elimination produced free thiols which can subsequently catalyze the reshuffling of intact protein disulfides and lead to intermolecular disulfide cross-linkage.[36]

Chemical instability in the solid state can also occur after proteins first experience physical instability, such as denaturation or unfolding. Lyophilized recombinant human keratinocyte growth factor (rhKGF) is prone to aggregation at elevated temperatures.[39] Its aggregation pathway is proposed to proceed initially with unfolding of the protein, leading to formation of large soluble aggregates, which can form disulfide bonds.[39] Finally, these precipitates are converted to scrambled disulfides and/or nondisulfide cross-linked oligomers. Recombinant human albumin (rHA) also undergoes intermolecular thiol−disulfide interchange.[40] The unfolding or loss of tertiary structure in lyophilized rHA is suspected to have initiated the covalent thiol−disulfide exchange.[40] The covalent aggregation of ovalbumin, glucose oxidase, and β-lactoglobulin have also been linked to initial protein physical instability, such as protein unfolding.[37]

Nonreducible Dimerization and Aggregation—Lyophilized human insulin can undergo covalent dimerization at the Asn_{A21} site to form [Asp_{A21}-Phe_{B1}] and [Asp_{A21}-Gly_{A1}] insulin dimers.[13] This dimerization occurs after deamidation of the C-terminal Asn_{A21} forms a reactive cyclic anhydride intermediate that further reacts with the free N-terminal amine of another insulin molecule to yield dimers.[13] Lyophilized tumor necrosis factor (TNF) is also susceptible to the formation of cross-linked aggregates consisting of dimers, trimers, and higher oligomers, which were nonreducible when treated with β-mercaptoethanol.[41]

Another protein that forms nonreducible dimers in the solid state is recombinant bovine somatotropin (rbSt). When lyophilized rbSt was stored at 30 °C and 96% RH, the rate of loss of monomeric rbSt was greater than or equal to the rate of loss in solution.[12] Covalent dimers accounted for 80−90% of the total degradation products observed, with the remaining 20−10% attributed to deamidation/cleavage. The fractional amount of nonreducible dimers was only 23−26% in solution.[12] Most of the covalent dimers were not reducible with β-mercaptoethanol, indicating that the covalent bonds were not disulfide bonds.[12] Hageman et al. hypothesized that such covalent interaction could be due to the condensation of a lysine ϵ-amine and a carbonyl side chain of either Asp or Glu.[12]

A similar mechanism was proposed for the degradation of lyophilized ribonuclease A due to the formation of soluble and insoluble aggregates during storage at 45 °C. These aggregates were not dissociable using an anionic detergent (sodium dodecyl sulfate) and a reducing agent (2-mercaptoethanol), indicating that the aggregates were covalently attached but not through disulfide bonds.[42] Originally, protein oxidation was believed to be the cause of the aggregation since exposure to oxygen and light increased the solid-state aggregation rate of ribonuclease A.[43] Upon further investigation, Townsend et al. postulated that these covalent linkages did not result from an oxidation reaction but from the lysine residues reacting with asparagine or glutamine in a manner similar to the mechanism proposed by Hageman et al. for rbSt.[44] The loss of free lysine residues in the insoluble aggregates, as determined by amino acid analysis, is consistent with this mechanism.[44]

A loss of lysine and histidine residues also was observed by Schwendeman et al. in studies of the moisture-induced aggregation of tetanus and diphtheria toxoids.[45] In this case, however, aggregation was thought to be caused by formaldehyde-induced cross-linking of the proteins, since the aggregates were not soluble under either reducing (10 mM dithiothreitol, 1 mM EDTA) or denaturing (6 M urea) conditions, and since the toxoids had been treated with formaldehyde ("formalinized") during production.[45] The authors proposed a mechanism for the formation of stable intermolecular cross-links in this system and established methods for stabilizing the toxoid formulation against this reaction. Effective stabilization methods included succinylation, treatment with sodium borohydride, and addition of sorbitol.[45]

Factors Influencing Protein and Peptide Chemical Instability in Solids

The importance of temperature, moisture, and formulation excipients in determining the solid-state stability of small molecule drugs has been widely reported and accepted.[45−47] However, the effects of these factors on the solid state chemical stability of proteins and peptides are not as widely reported or understood. In addition to these factors, there is a growing literature that suggests that the hydrogen ion activity in the solid-state may have a significant influence on peptide and protein stability, paralleling the influence of pH in the solution state. The physical state of the solid (e.g., glassy vs rubbery) also has been shown to affect protein reactivity in the solid state. This section reviews published reports of the effects of these factors on peptide and protein stability in the solid state: temperature, moisture, excipients, hydrogen ion activity, and the physical state of the solid.

Temperature—The exposure of solid protein and peptide formulations to elevated temperatures generally decreases chemical stability by accelerating almost all chemical degradation reactions. For example, the deamidation of the Asn-hexapeptide increases with increasing temperature in the solid-state, with the formation of the cyclic imide intermediate becoming more favored over the direct hydrolysis of the Asn side chain.[18] In general, the increase in degradation rates with temperature did not follow an Arrhenius relationship within the temperature range studied (40−70 °C).[18] This suggests that acceleration of chemical reaction kinetics due to temperature is not solely responsible for the rate increase.

High-temperature inactivation of proteins often results from the destruction of disulfide bonds in cystine via β-elimination.[1] Consistent with this, the aggregation of insulin, in which β-elimination is the first step, was observed to increase with increasing temperature.[36] Increases in temperature have been observed to increase the rate of covalent cross-linking in the solid state for rbSt,[12] bovine insulin,[36] and tumor necrosis factor.[41] Most degradation reactions, such as deamidation,[18] peptide bond cleavage,[23,26] the Maillard reaction,[33] and oxidation[49] are accelerated by increasing temperature.

The increases in chemical instability with temperature may be due to increased mobility in the system or lowering of the activation barrier for reaction. In particular, significant changes in mobility may occur when the temperature range of interest includes the glass transition temperature (T_g) of an amorphous solid. In this case, changes in temperature affect not only the intrinsic reactivity of the degrading species, as observed in the solution state, but also the medium in which the reaction occurs. The relationship between the glass transition temperature (T_g) and reactivity is discussed below, under "Physical State of the Solid".

Moisture—Residual moisture is often thought to be responsible for protein and peptide chemical instability in the solid-state. In general, lyophilized protein formulations are more stable at lower water contents.[2] For example, the rate of degradation of the Asp-hexapeptide increases as the formulation vial moisture content increases.[23] Likewise, the deamidation of the Asn-hexapeptide increases as water content increases from 0.5% to 2%.[18] However, further increases in water content of up 3% do not significantly affect the rate of degradation.[18]

A similar effect of water on the deamidation of insulin was observed. The rate of degradation of lyophilized human insulin increases steeply with water content at low levels of hydration, approaching apparent plateaus at water contents greater than 20%.[13] The rate constants in the plateau region are comparable to those in solution.[13] The rate of rbSt loss due to deamidation and dimerization also increased with increased water content.[12] Increasing the relative humidity from 75% to 96% doubled the rate of rbSt loss.[12] However, the fractional amount of nonreducible dimers formed remained constant at around 80% and was independent of water content over the range studied.[12]

The covalent aggregation of bovine serum albumin (BSA) in the solid state greatly depends on water content.[38] Freeze-dried BSA was more prone to aggregation in the moistened solid state than in solution and exhibited a maximum at a very low moisture level.[38] The aggregation of BSA in solution increased with increasing ratio of water to protein.[38] Jordan et al. proposed that the increase in aggregation rate is related to the increase in water mobility with increasing ratios of water to protein.[38] Lyophilized recombinant human interleukin-1 receptor antagonist (rhIL-1ra) formulated with 2% glycine, 1% glycerol, and 10 mM citrate buffer at pH 6.5 exhibited maximum relative instability at a moisture content of around 0.8% (w/w) and the least stability at water contents less than 0.5% or around 3.2% (w/w).[11] A similar bell-shaped relationship between covalent aggregation and water content has been observed for ovalbumin, glucose oxidase, and β-lactoglobulin.[37]

A few other types of chemical instabilities appear to exhibit this bell-shaped relationship between degradation kinetics and moisture content. A bell-shaped dependence on moisture content also was observed for the aggregation of rHA via thiol-disulfide exchange, with maximum aggregation corresponding to 47 g water/100 g dry protein (96% RH).[40] The Maillard reactions usually have maximum rates in the low-moisture range.[34] Insulin,[50] casein,[51] and blood plasma proteins[52] in "dry" systems containing glucose show a maximum in the range of 40−80% RH (10−20% water content).[2]

This bell-shaped relationship between water content and reaction rate is often attributed to the effect of water on molecular mobility and reactant concentration. At low moisture levels, the Maillard reaction is less rapid because the diffusion and mobility of reactants are restricted.[2,34,52] As water content increases, molecular mobility increases, leading to enhanced reactant mobility which should facilitate the reaction.[37] At higher water contents, the reaction rates decrease due to dilution of the reactants by water.[37,53] Liu et al. observed that the addition of various types of polymers (carboxymethyl-cellulose, dextran, poly(ethylene glycol)) as diluents also appeared to hinder the aggregation of BSA.[37]

While residual water can often increase chemical degradation rates, under some conditions it can suppress a chemical reaction. Pikal et al. observed that the oxidation of a lyophilized hGH formulation was affected by increases in headspace oxygen at low moisture levels but not at higher moisture contents.[29] This may be due to water's possible antioxidant properties. Water may be either an antioxidant or an oxidant depending on the system.[32] Generally, oxidation in foods reaches a maximum at low water content and decreases as water content increases.[2]

In addition to having an effect on solid-state degradation rates, water may also affect the reaction pathways. For example, the formation of the cyclic imide intermediate during deamidation of the Asn-hexapeptide at pH 5 was favored over direct hydrolysis as water content increased.[18] The low moisture environment of the solid-state can generate product distributions which differ from those of the solution state.[18] The main product in the degradation of the Asp-hexapeptide in solids lyophilized from pH 3.5 and 5 solutions is the cyclic imide hexapeptide.[23] In contrast, the dominant degradation product in solution at pH 3.5 is the tetrapeptide and at pH 5.0 is the isoAsp-hexapeptide.[24] Thus, the reduced moisture in the lyophilized solids appears to suppress the hydrolysis of the Asu-hexapeptide intermediate to form the isoAsp-hexapeptide and that of the Asp-Gly peptide bond to form the tetrapeptide.

We have observed similar shifts in the product distribution in our recent studies of Asn-hexapeptide deamidation in poly(vinyl pyrrolidone) and poly(vinyl alcohol) matrices.[21] In addition, the order of the reaction with respect to water content differs in these two polymers: it is first-order in poly(vinyl pyrrolidone), but second order in poly(vinyl alcohol).[21] Since it is unlikely that the molecular deamidation mechanism differs significantly in the two systems, the results suggest that the role of water extends beyond that of reactant and may involve plasticization of the polymer matrices.

So, while the importance of water in chemical reactions is well recognized, the exact relationship between solid-state chemical instability and water content is often difficult to delineate and is dependent on the type of degradation reaction(s). This difficulty in determining the mechanism by which water affects the chemical stability of proteins and peptides in the solid state is in part due to the various roles water can play in promoting chemical reactivity. Water can act as (1) a reactant, (2) a medium or solvent, and (3) a plasticizer to increase molecular mobility.[2,47,54] Discriminating among these possible effects is difficult, particularly since more than one effect may be important in a given system.

Excipients—Proteins are often formulated with excipients, such as sugars and polymers, to protect them during

Table 1—Types of Polyalcohols Used as Pharmaceutical Excipients

polyhydric alcohols	nonreducing sugars	reducing sugars
mannitol	dextran	fructose
sorbitol	sucrose	glucose (dextrose)
	trehalose	lactose
		maltodextrin
		maltose

freeze-drying and storage. Polymeric "excipients" may also be used to form a matrix for controlled release. The types of excipients used and their effects on the chemical stability of proteins and peptides are varied. For example, a wide range of excipients, such as heparin, sulfated polysaccharides, anionic polymers, and citrate, decreased the rate of covalent aggregation in recombinant human keratinocyte growth factor (rhKGF) at elevated temperatures.[39] On the other hand, while EDTA inhibited disulfide formation in aggregates, it was not as effective in stabilizing rhKGF.[39] In this section, three categories of excipients will be discussed since they comprise the majority used in solid formulations: polyalcohols, polymers, and buffer salts.

Polyalcohols—Sugars and polyols are the most commonly used excipients in lyophilized protein and peptide formulations.[55] Table 1 lists the different types of polyalcohols used as pharmaceutical excipients. These sugars and polyols are often added to lyophilized formulations to protect and stabilize the proteins against decomposition during processing and storage. TNF formulated with dextran, sucrose, trehalose, or 2-hydroxypropyl-β-cyclodextrin, which are amorphous, formed fewer nonreducible dimers than TNF formulated without an excipient.[41] The covalent dimerization of human insulin was markedly decreased by incorporation into a glassy matrix of trehalose.[56] The stability of lyophilized recombinant human interleukin-1 receptor antagonist (rhIL-1ra) was greatly improved by increasing the concentration of sucrose in the formulation.[11] Sucrose stabilized rhIL-1ra against chemical degradation better than trehalose.[11] Examination of a series of polyalcohols in this system showed the following order of decreasing ability to stabilize the protein: sucrose > trehalose > sorbitol > no excipient > maltose.[11] In a recent study of Asp-Pro bond cleavage in Physalaemin and Hamburger peptides, stabilization was provided by glassy polyols in the order: sucrose > Ficoll (low mw) > Byco A, Ficoll (high mw) > dextran.[57] The authors attributed the stabilizing ability of the polyols to the density of the matrix, with greater packing density leading to improved stability.

Some of these excipients, such as maltose in the rhIL-1ra example above, may have a destabilizing effect. For example, the oxidative deamidation of a cyclic heptapeptide was observed in formulations containing mannitol, but not in solution or in the neat solid (no excipient).[31] The extent of oxidative degradation increases with increasing mannitol content after 12 weeks of storage at 40 and 60 °C.[31] These researchers postulated that the cyclic heptapeptide was reacting with a reducing sugar impurity in the mannitol.[31] Thus, the lower stability of the rHIL-1ra maltose formulation may have been due to the rhIL-1ra reacting with maltose, a reducing sugar.

Reducing sugars are known to react with proteins and peptides via the Maillard reaction (formation of Schiff base) as well as other side reactions.[2,25,55] Rapid covalent modification of lyophilized human relaxin was observed in the presence of glucose.[25] The glucose appeared to have reacted with lysine and arginine residues via the Maillard reaction to form covalent adducts.[25] A significant amount of serine cleavage from the C-terminal of the B-chain of relaxin was also identified.[25] A high glucose content may be necessary for cleavage to occur, since the hydrolytic cleavage of relaxin stored in concentrated glucose solutions occurred to a lesser extent than in the solid state.[25] In contrast, formulating relaxin with nonreducing sugars such as mannitol and trehalose produced stable, lyophilized formulations.[25]

In addition to oxidation and deamidation products, human growth hormone formulated with lactose produced an additional product not observed in either the freshly freeze-dried lactose formulation or in any of the other formulations studied. The researchers postulated that the product was due to an adduct of lactose and hGH, either the glycosylamine or the corresponding Schiff base.[4] As stated above, the use of reducing sugars in lyophilized formulations is problematic due to their propensity to react with proteins via the Maillard reaction, especially at lysine and arginine residues.

Polymers—Polymeric excipients may influence the reactivity of peptides and proteins through direct chemical interactions or by altering the physical state (e.g., glassy vs rubbery) of the system. This section addresses chemical interactions between polymeric excipients and incorporated proteins. The effects of polymers on the physical state of the matrix are discussed below.

Many of the published studies on the chemical effects of polymers on peptides and proteins involve the lactide/glycolide copolymers. These polymers are of particular interest because they are biocompatible and biodegradable and are therefore used commonly in implantable devices.

The stability of atriopeptin III (APIII) encapsulated in poly(D,L-lactide-*co*-glycolide) (PLG) microspheres was studied and compared to that of APIII alone at 40 °C.[58] When microspheres were shaken in neutral buffer solution, APIII was completely degraded in 5 days. In neutral buffer alone, APIII degraded with a half-life of 8.7 days.[58] After 8 days at 95% relative humidity, 20% of APIII in the microspheres had degraded.[58] Concentrated APIII showed no degradation after 2 weeks, and dilute APIII in water showed no loss after one month.[58] Different degradation peaks were observed for APIII in microspheres and APIII alone.[58] It was concluded that PLG catalyzes the degradation of APIII, and the mechanism of this degradation appears to be different from that seen for APIII alone in buffer.[58] This suggests that the polymer environment has a significant effect on the chemical stability of this protein.

Park and co-workers also found this to be the case for the stability of carbonic anhydrase and bovine serum albumin during release from a polymer matrix immersed in buffered medium at 37 °C.[59] Carbonic anhydrase encapsulated in poly(D,L-lactic acid-*co*-glycolic acid) (PLGA) microspheres was released in an inactive form.[59] Severe hydrolysis of unreleased and released proteins was observed.[59] It was postulated that the hydrolysis was due to an acidified medium created by the degradation of the PLGA microspheres.[59] The degradation of the PLGA microspheres occurs via random chain scission of an ester linkage in the polymer backbone, which produces water soluble oligomers with carboxylic acid end groups that affect medium pH.[59] As the microspheres degraded, the pH of the medium decreased from 7 to 3 over 40 days.[59] Park et al. also suggest that the protonated carboxylic acid end group of the lactic and glycolic acid fragments may react with the carbonyl group of the amide linkage in the proteins, leading to cleavage of the peptide backbone.[59] So, in addition to having an environmental effect, polymers may also have a direct catalytic effect on the chemical stability of proteins in solids.

In contrast to these reports of a detrimental effect of lactide-glycolide polymers on protein stability, there have been several reports of successful protein formulation in

these polymers.[60−62] Cleland et al. describe the stability of recombinant human growth hormone (rhGH) during release from PLGA microspheres into isotonic buffer.[60] Rates of oxidation, diketopiperazine formulation, and deamidation in the microspheres were comparable to those in solution controls, and rates of aggregation were only slightly greater than in solution.[60] The authors infer that the pH within the microspheres is comparable to that in the surrounding buffer, and that rapid buffer equilibration may prevent a pH decrease in this system.[60] In related studies, rhGH was stabilized in a PLGA microsphere formulation by the formation of an insoluble complex with zinc.[61] In vitro studies showed that microsphere encapsulation did not alter rhGH activity in this system, and in vivo studies in rats and monkeys showed sustained delivery of rhGH for up to one month.[61] The discrepancies in protein stability in PLGA systems suggest that factors other than the polymer choice will influence drug stability. These may include the properties of the protein drug itself, other formulation variables, and the interaction of the formulation with the release matrix (e.g., buffer, tissue).

Limited information is available on the chemical effects of other polymeric excipients on peptide and protein stability. Recent studies in our laboratories suggest that the Asn-hexapeptide forms a reversible complex with poly(vinyl pyrrolidone) when stored in matrixes of this polymer at elevated temperature and low matrix moisture content.[63] Formation of the complex results in a reduction in Asn-hexapeptide degradation to the cyclic imide, but release of the Asn-hexapeptide from the complex requires more than 1 week in dialysis studies at neutral pH.[63] The chemical nature of this complex is under investigation. Protein binding to dextrans in lyophilized proteins has also been suspected.[64] After exposure to aqueous media for more than 2 weeks, recombinant bovine somatotropin (rbSt) and zinc insulin were released intact and active from microspheres composed of sucrose and poly[1,3-bis(*p*-carboxyhydroxy)-hexane anhydride], which is a very hydrophobic polymer.[65] Ron et al. postulated that the hydrophobic polymer prevented the entry of water into the microspheres[65] and thus protected rbSt from moisture induced decomposition, such as covalent dimerization and deamidation.[12]

Buffer Salts—Buffer salts are often included in solid formulations of peptides and proteins in an attempt to control the hydrogen ion activity, or the protonation state of the functional groups on the protein. In solution, buffer salts may have a catalytic effect on many protein degradation reactions, apart from their effect on pH. In this section, we review the limited evidence for specific chemical effects of buffers in the solid state. The effects of solid-state hydrogen ion activity on reactivity are discussed in the next section.

There is some evidence that phosphate buffers have a catalytic effect on protein degradation. In a study on lyophilized RNase, Townsend and DeLuca prepared lyophilized formulations from solutions containing varying concentrations of sodium phosphate buffer.[66] While a buffer-free formulation showed a 10% loss in activity over 120 days of storage at 45 °C, a formulation lyophilized from a 0.2 M buffer solution showed a nearly 40% loss in activity during the same period.[66] Similarly, Pikal et al. showed an effect of sodium phosphate buffer concentration on the chemical degradation and aggregation of lyophilized human growth hormone.[4] Mechanisms suggested for this effect include the catalysis of oxidation reactions by heavy metal contaminants in the buffer salts,[66] and the preferential crystallization of phosphate buffer components during freeze-drying, leading to pH shifts.[4] It should be noted that sodium phosphate buffers are thought to be particularly prone to preferential crystallization, and often are avoided in lyophilized protein formulations for this reason.[67] Acetate buffers are also avoided in lyophilized formulations, since volatilization of acetate species during freeze-drying may alter the buffer composition in the solid.

Buffer species themselves may be chemically unstable in solid formulations, producing degradation products that interact with peptides and proteins. Recent studies in our laboratories examined the deamidation of the Asn-hexapeptide in lyophilized matrixes containing PVP and Tris buffer.[68] We observed the formation of a new peak in HPLC chromatograms, which mass spectral analysis showed to be a formaldehyde adduct of the tyrosine residue on the aspartate or isoaspartate deamidation product. The presence of formaldehyde was shown to be associated with the inclusion of Tris buffer in the formulation, since no formaldehyde was detected when Tris was omitted or replaced with other buffers.[68] We hypothesize that degradation of Tris [tris(hydroxymethyl)aminomethane] occurs on storage of this formulation, liberating formaldehyde, which then reacts with the tyrosine residue on the peptide.[68]

Hydrogen Ion Activity—In the solution state, pH is used as a simple indicator of hydrogen ion activity. While pH is not defined in the solid state, there is a strong correlation between the chemical stability of proteins and peptides in the solid state and the pH of the solution prior to lyophilization. This value is often termed the "apparent pH" of the solid and is often given the abbreviated designation, "pH", with the quotation marks signifying that the value is apparent.

The deamidation rate of the Asn-hexapeptide increases as the solution pH prior to freeze-drying increases from 5 to 8.[18] The "pH" of the Asn-hexapeptide formulation solution also appears to dictate the extent to which the peptide stability depends on other variables, such as moisture level and temperature.[18] The deamidation of lyophilized human insulin is also quite sensitive to "pH".[13,14] Increases in the oxidation of hGH,[4,29] the β-elimination of cysteine in bovine insulin,[36] and the formation of nonreducible dimers of tumor necrosis factor[41] in the solid state all increase with formulation "pH". Interestingly, while no dimers were observed at "pH" 4, the biological activity of TNF at "pH" 4 was significantly lower than at higher "pH's".[41]

The increase in the rate of covalent dimerization of rbSt with increasing "pH" is attributed to the deprotonation of the lysine residues, which are more nucleophilic when deprotonated. Interestingly, the fractional amount of nonreducible dimers formed during rbSt degradation remained constant at around 80%, independent of "pH" over the range studied (pH 5−10).[2,69] Lyophilized recombinant human interleukin-1 receptor antagonist (rhIL-1ra) formulations containing citrate were less likely to form noncovalent aggregates than those with phosphate.[11] The researchers postulated that the destabilizing effect of phosphate buffer may be due to the selective crystallization of the dibasic salt, which would expose the protein to a low "pH".[11]

To avoid drastic pH shifts, the weight ratio of buffer to other solutes should remain low.[3] The addition of ionic agents such as NaCl should also be kept at a minimum or avoided. While NaCl does not affect "pH", the chemical stability of hGH in freeze-dried formulations was decreased by the presence of NaCl.[4] The researchers attributed the destabilizing effect of NaCl on hGH to a specific ion effect, due primarily to the chloride ion.[4] The effect of NaCl on hGH may also be due to a salt effect on "pK_a".

The Physical State of the Solid—The state of a pharmaceutical solid depends on the temperature, composition (drug and excipient), moisture, and on the time allotted to the experiment. While the effects of tempera-

ture, moisture, and formulation composition on chemical instability were presented independently above, their influences on the solid-state chemical stability of proteins and peptides are often synergistic. These variables combined determine the physical bulk state of solid protein and peptide formulations. The bulk state of a formulation (crystalline versus amorphous) has been linked to various cases of protein and peptide chemical instability in the solid-state.[4,13,18]

Crystalline—Crystalline solids possess long range molecular order with the molecules structured in fixed geometric units. In contrast, amorphous solids have irregular packing and lack long range order. For small molecule drugs in the solid-state, the crystalline drug is generally less prone to chemical decomposition than the amorphous form.[14,70] However, the crystalline state may not be more stable for protein and peptide formulations. Oliyai et al. found that the Asp-hexapeptide was more stable in a lactose matrix (amorphous) than in mannitol (crystalline).[23] Interestingly, the lactose formulation had a greater affinity for water (a higher water content) than the mannitol formulation, yet the peptide was significantly more stable in the lactose matrix than in mannitol.[23] Excipients that were more hygroscopic were generally better at stabilizing rHA against aggregation, suggesting that the stabilization arises primarily from interactions between the excipient and water rather than between the excipient and the protein.[40]

The stability of crystalline and amorphous insulin (neat solid) was also investigated at various water contents. For crystalline insulin, deamidation at the Asn_{A21} site increases rapidly with increases in water content.[14] In contrast, the deamidation of amorphous insulin was nearly independent of moisture content (up to 15%).[14] Crystalline human insulin was less stable than amorphous insulin, independent of the water content over the range studied.[14]

Solid formulations which are at least partially amorphous appear to best protect proteins and peptides against chemical instability.[4] Lyophilized human growth hormone (hGH) formulated in a partially amorphous excipient system (glycine:mannitol) was less susceptible to chemical degradation and aggregation than hGH formulated in either totally amorphous (dextran) or crystalline (mannitol) systems.[4] So, while a partially amorphous system is necessary to stabilize hGH, an amorphous system is not a sufficient condition for stability.[4] Pikal et al. postulated that a partially amorphous system allows molecular interaction with the protein and/or acts as "sink" for residual water.[4]

While residual moisture generally leads to chemical instability, the relative stability of proteins and peptides in solid formulations cannot be judged solely on the basis of the amount of water present, as seen in the above cases. The nature of the excipient, such as its crystallinity and affinity for water, appears to be important as well.

Amorphous—Depending on temperature, structure, and composition, amorphous solids such as polymers exhibit widely different physical and mechanical properties.[71] At low temperature and moisture, amorphous solids are brittle, hard, and highly viscous, and are said to be in the "glassy" state. In the glassy state, molecular motion is largely restricted to vibrations and short-range rotational motion.[71] As the temperature and/or moisture content increases, the solids undergo a transition from a glassy state to the so-called "rubbery" state, which is characterized by decreased viscosity and increased elasticity. The glass transition temperature (T_g) is defined as the temperature at which the solid softens because of the onset of long-range coordinated molecular motion.[71] The T_g varies greatly among different amorphous pharmaceutical solids and is dictated by such factors as composition and moisture content, since water may act as a plasticizer to increase bulk mobility and decrease T_g.

Researchers have speculated that chemical reactions which involve molecular mobility, such as bimolecular reactions, are unlikely to occur in the glassy state due to the limited mobility.[72] As the solid system becomes more rubbery ($T > T_g$) and mobility increases, the reaction rates would then greatly increase.[72] Lyophilized rhIL-1ra formulations with T_g's ranging from 20 to 56 °C were stored for several weeks at temperatures above and below their T_g's.[73] The decomposition of rhIL-1ra, both via deamidation and aggregation, was greatly accelerated at temperatures above T_g (rubbery state).[73] However, the rate of degradation also increased in some formulations whose T_g's were above the storage temperature (glassy state).[73]

Other instances of protein and peptide degradation in the glassy state have been documented. The aggregation of hGH has been found to occur in formulations stored at temperatures well below their glass transition temperature.[4] The dimerization and deamidation of insulin have been observed in the glassy state;[13] however, the actual formulation T_g was not measured in this study. Recently, the fission of the Asp-Pro bond in Physalaemin and Hamburger peptides has been detected in water soluble glasses (e.g., dextrose, Ficoll, sucrose) below their T_g.[57] Thus, while proteins and peptides are generally more stable in the glassy state, chemical degradation reactions can still occur. Although mobility is limited in the glassy state, glassy pharmaceutical solids may experience significant molecular mobility at temperatures up to 50 °C below their glass transition temperature.[74] Furthermore, significant rotational and translational mobility was shown to exist for water in solid poly(vinyl pyrrolidone), even at very low water contents (glassy state).[75]

The increase in reactivity with water content is often attributed to water's ability to plasticize or increase the molecular mobility of the bulk structure and thus the reactants. The decomposition of a freeze-dried monoclonal antibody–vinca conjugate has been correlated with formulation T_g.[76] However, since water activity and content were not kept constant, other factors which may contribute to this instability cannot be ruled out. In contrast, the degradation of aspartame in the solid-state correlates better with water activity and content than with T_g.[77] In recent studies of Asn-hexapeptide deamidation in poly(vinyl pyrrolidone) matrices, we have attempted to discriminate between water's effects on T_g (i.e., as a plasticizer) and its solvent effects by adding glycerol to the matrix as a secondary plasticizer.[22] This enabled us to generate systems with constant T_g and varying water activity, or with constant water activity and varying T_g. Interestingly, the deamidation rate showed a single log-linear correlation with T_g for matrices in the rubbery state, while in the glassy state this correlation resulted in multiple parallel lines with intercepts dependent on matrix water activity.[22] These results suggest that for this system, water acts primarily as a plasticizer in the rubbery state, but acts both as a plasticizer and a solvent or reaction medium in the glassy state. Taken together, these conflicting reports on water's role as a plasticizer demonstrate the complexity of these systems and the attendant difficulty in gaining a mechanistic understanding of the chemical reactivity of incorporated proteins and peptides.

Summary and Commentary

The results discussed above highlight the difficulty in determining the mechanisms of protein chemical instability in the solid-state. Protein decomposition in the solid state

can occur through multiple degradation pathways, involving both physical and chemical instabilities, as observed for rbSt and insulin. Since chemical instability can also lead to physical instability and vice versa, it can be difficult to determine the mechanism of chemical degradation in proteins. Furthermore, non-peptide/protein components of these solid-state formulations may have multiple effects. Residual moisture and polymeric excipients, for example, may influence matrix mobility (T_g), may participate directly or indirectly in degradation reactions, and may influence the "pH" or other properties of the reaction medium. Multiple effects caused by a single additive make mechanistic definition difficult.

Consideration of the available literature in this area suggests several avenues for additional research. Most notably, it is clear that the determinants of protein and peptide reactivity in the solid-state have not been fully characterized. While pH, temperature, and buffer composition are known to be critical in solution, different or additional factors may be important in the solid-state. These may include mobility in the matrix, hydrogen ion activity, and protein secondary and tertiary structure. Additional research relating to each of these factors is warranted.

For example, while mobility in a solid polymer matrix is often characterized using the glass transition temperature (T_g), this measurement describes the mobility of the matrix as a whole and not its specific components. Ultimately, the degradation of peptides and proteins in these systems is more likely to depend on the mobility of the drug molecules themselves or of specific amino acid residues within them, rather than on the "bulk" mobility described by T_g, particularly when the drug loading is low. Recent research has begun to address this question,[78,79] but continued effort will be required to establish a measure of solid-state mobility that is predictive of reactivity. In addition, mobility is likely to be more important for bimolecular reactions involving large molecules (e.g., protein aggregation) than for unimolecular reactions (e.g., succinimide formation) or bimolecular reactions involving relatively small, mobile reactants (e.g., oxidation). An understanding of the factors that produce a "mobility-limited reaction" in the solid state would aid in formulation, since stabilization strategies that reduce mobility (e.g., inclusion of glass-forming excipients) are most likely to be effective for these reactions. Reaction mechanism and reactant molecular weight are likely to be among these factors.

Hydrogen ion activity (i.e., pH) is important in the solution state degradation of many peptides and proteins, but its role in the solid-state remains unclear. At present, there is no convenient measure of hydrogen ion activity in these solid systems, so examination of its effects has relied on correlation with "effective" pH. These studies have suggested that the "pH" dependency in the solid state parallels its solution state counterpart. However, it is reasonable to expect that a reaction that is pH-driven in solution may be controlled by other factors, such as mobility, in a glassy solid. Examination of the dependence of solid-state "pH" rate profiles on other matrix properties would be useful, as would the development of methods to measure hydrogen ion activity in solid formulations.

Finally, the effects of protein secondary and tertiary structure on reactivity and reaction mechanism should be examined. In solution, secondary structure has been shown to affect deamidation rate,[80] and protein aggregation in the solid state has been attributed to adsorption-induced unfolding.[62] In addition, a recent study has demonstrated the utility of infrared spectroscopy to determine protein secondary structure in PLGA matrixes.[81] However, there is a lack of information on the effects of protein structure on chemical reactivity in solids. Such information is important not only for chemical routes of protein degradation, but also because physical modes of degradation, such as aggregation, are often associated with chemical changes. Information on the interaction of protein structural domains with matrix properties (such as mobility and water content) to produce degradation would help to identify labile residues in solid protein formulations a priori. Stabilization strategies could then be directed in a rational way toward the modification of protein structure and/or formulation variables.

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