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Expert Review

Stability of Protein Pharmaceuticals: An Update

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Abstract. In 1989, Manning, Patel, and Borchardt wrote a review of protein stability (Manning *et al*., Pharm. Res. 6:903–918, 1989), which has been widely referenced ever since. At the time, recombinant protein therapy was still in its infancy. This review summarizes the advances that have been made since then regarding protein stabilization and formulation. In addition to a discussion of the current understanding of chemical and physical instability, sections are included on stabilization in aqueous solution and the dried state, the use of chemical modification and mutagenesis to improve stability, and the interrelationship between chemical and physical instability.

KEY WORDS: formulation; protein stability; protein stabilization.

INTRODUCTION

In 1989, Manning, Patel and Borchardt wrote a review summarizing what was known at the time about the stability and stabilization of protein pharmaceuticals (1), an article that has been referenced almost 500 times. In the late 1980s, there were only three recombinant protein products on the US market: Orthoclone (OKT-3), human insulin, and tissue plasminogen activator. If one included plasma-derived products, the number of approved proteins only numbered about a dozen. Clearly, recombinant DNA technology has drastically changed the pharmaceutical market. Now there are nearly twenty antibody products and almost 150 approved protein-based products that are commercially available in the US alone. In addition, our knowledge regarding protein stability and formulation has increased dramatically. The purpose of this review is to provide an update regarding what we have learned in the past 20 years. In addition to updating the sections of the original review article, some discussion is provided regarding topics that were not found in the literature at the time, such as the interrelationship of chemical and physical instability, instabilities that occur during bioprocessing, the impact of lyophilization cycle on protein stability, and the importance of packaging in maintaining protein stability.

One can separate protein instabilities into two general classes: chemical instability and physical instability. Chemical instabilities involve processes that make or break covalent bonds, generating new chemical entities. A list of the more commonly observed chemical degradation processes is listed in Table I. Conversely, there are physical instabilities for proteins in which the chemical composition is unaltered, but the physical state of the protein does change. This includes denaturation, aggregation, precipitation, and adsorption (Table I). The term precipitation is used here to denote insolubility rather than insoluble aggregate formation.

Our knowledge of all protein degradation pathways is markedly greater than it was 20 years ago. Therefore, the emphasis of this review is on the progress that has been made since 1989. In addition, there were degradation processes and topics that were barely discussed or observed at that time. Those are now included as separate sections below. For example, there have been many articles on increasing conformational stability of proteins with various excipients, both in aqueous solution and in the dried state. In addition, a brief overview is provided of protein stabilization methods, including various drying methods, chemical modification, and site-directed mutagenesis. Finally, a discussion of the interrelationship between chemical and physical instability is provided.

CHEMICAL INSTABILITY

Deamidation

Twenty years ago, it was already appreciated that deamidation, which involves the hydrolysis of Asn and Gln side chain amides, was a common degradation pathway for proteins and peptides. It is still regarded as the most common chemical degradation pathway for peptides and proteins. From a regulatory perspective, deamidation generates process-related impurities and degradation products. In addition, it may contribute to increased immunogenicity (2).

At the time of the original review article, there were a few examples of deamidation in pharmaceutically relevant proteins, including human growth hormone (hGH) (3,4), insulin (5), γ-globulin (6), and hemoglobin (7). Moreover, the effect of extrinsic factors, such as pH, temperature, and ionic strength, were known as well (8). Since that time, the amount of information now available on deamidation and related

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Table I. Chemical Instabilities Reported for Proteins of Therapeutic Interest

Deamidation
Asp–isoAsp interconversion/isomerization
Racemization
Proteolysis
Beta-elimination
Oxidation
Metal-Catalyzed Oxidation (MCO)
Photooxidation
Free radical cascade oxidation
Disulfide exchange
DKP formation
Condensation reactions
pGlu formation
Hinge region hydrolysis
Trp hydrolysis

reactions has increased significantly, as can be found in a number of excellent review articles (9–12) as well as entire books on the subject (13,14). There is even a web site devoted to this topic (www.deamidation.org).

Asn Deamidation

For those unfamiliar with this reaction, deamidation of Asn residues under acidic conditions takes place by direct hydrolysis of the Asn side chain amide to form only Asp. Under these conditions, deamidation is subject to acid catalysis. Similarly, Gln residues are converted to Glu (as is described in more detail below). However, this mechanism is rarely observed, as the pH must be less than 3. In neutral to basic solution (i.e., above pH 6), the mechanism changes to an intramolecular cyclization reaction. The first step involves nucleophilic attack of the *n*+1 nitrogen of the protein backbone on the carbonyl group of the Asn side chain (Fig. 1). This step is base catalyzed, since abstraction or partial abstraction of the backbone amide proton makes the nitrogen more nucleophilic, accelerating the reaction. A cyclic imide (also called succinimide or Asu) intermediate is formed (Fig. 1) with loss of ammonia. Since ammonia is a gas and is typically not retained in solution, this step is effectively irreversible. While the Asu intermediate often can be detected as a degradation product in its own right (see below), it is readily hydrolyzed in aqueous solution to form the Asp and isoAsp products (Fig. 1). Formation of the Asu five-membered ring intermediate is thought to be the reason that Asn deamidation is more prevalent than Gln deamidation, as five-membered heterocyclic rings are more stable than the six-membered rings associated with Gln deamidation.

Consequently, deamidation generates two degradation products (Asp and isoAsp) at the site of the original Asn residue. Coupled with the possibility for racemization (15), four possible products (L-Asp, D-Asp, L-isoAsp, and D-isoAsp) could be formed. It is now known that racemization does not occur to any appreciable extent from the Asu intermediate, as was previously thought. Instead, it appears to be a parallel degradation pathway (16). Dehart and Anderson have provided a detailed kinetic description of the intramolecular cyclization (17). The same observation of a lack of racemization via the cyclic imide intermediate has been made for larger proteins as well (18).

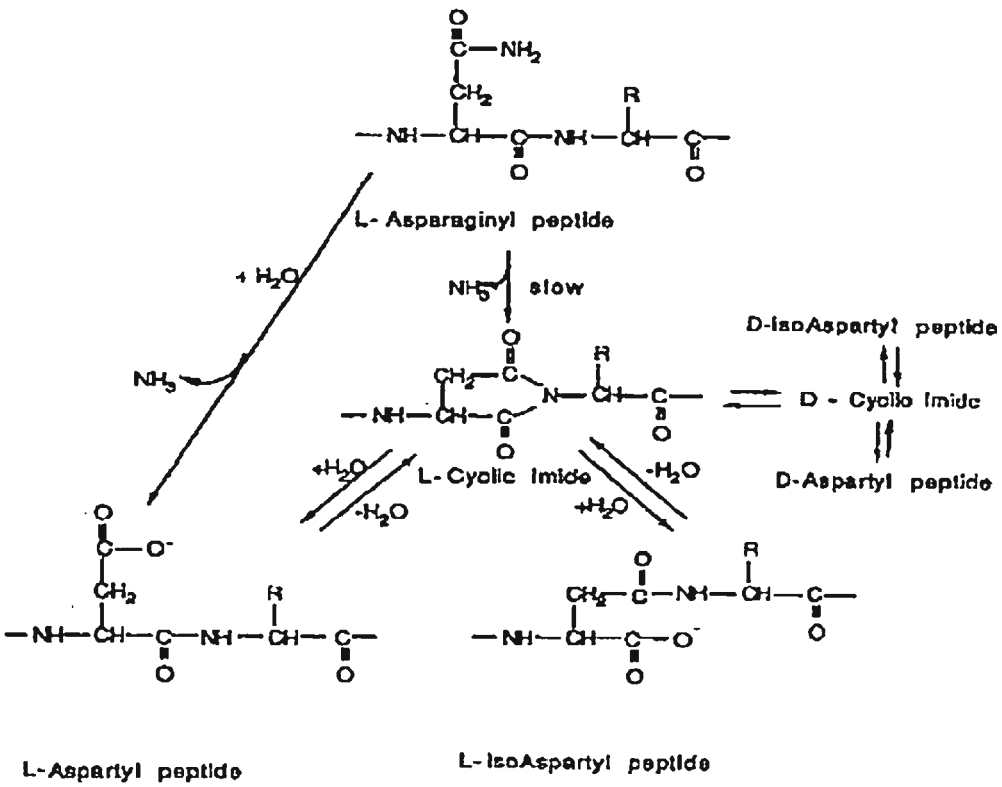

Fig. 1. General mechanism for deamidation of Asn residues and isomerization of Asp to isoAsp (taken from reference 1). Direct hydrolysis occurs below pH 4 while the cyclic imide pathway predominates at pH 6 and above.

Effect of Sequence on Asn Deamidation

Some work had been done by 1989 on the effects of primary sequence on deamidation, especially recognizing that Asn–Gly were particularly prone to deamidation. Subsequently, the influence of sequence was examined by Robinson and coworkers in detail (19,20). Ultimately, their work on sequence effects resulted in effective predictive schemes (14,21–25). In general, two trends are apparent. First, having amino acids with smaller side chains after the Asn residues leads to faster deamidation, presumably due to lack of steric hindrance of the initial cyclization reaction. Second, succeeding amino acids that have side chains that can act as hydrogen bond donors tend to accelerate the reaction, likely due to intramolecular hydrogen binding to the carbonyl oxygen of Asn, making it more electrophilic and thereby more reactive to nucleophilic attack.

As a result, one does not need to be concerned about deamidation at every Asn residue. Only those Asn residues followed by a small or hydrogen bond-donating (e.g., Ser, Asn, or Asp) residues are found to exhibit deamidation on a time scale relevant to the pharmaceutical scientist. For example, Chelius *et al.* found that Asn deamidation in monoclonal antibodies (MAbs) occurred at Asn–Gly and Asn–Asn sequences (26), while Xiao and Bondarenko found deamidation at Asn–Asp sequences (27). Overall, Asn–Gly is the most reactive sequence in polypeptides, consistent with the schemes of Robinson and Robinson (Table II). For the most part, the preceding residue has little or no effect on deamidation rate, at least in solution. However, Li *et al.* have shown that Gln or Glu in that position appears to accelerate deamidation in the solid state, presumably by increasing hydration around the Asn residue (28).

For deamidation that occurs at acidic pH, the mechanism does not involve cyclic imide formation at all. Instead, the protonated amide side chain undergoes direct nucleophilic attack by water. Therefore, it is not surprising that sequence has been found to play a minimal role in controlling deamidation rates (29).

Table II. Relative Deamidation Rates for Asn–Xaa where Xaa is the Succeeding Amino Acid (Taken from Reference 21)

Residue	% deamidation after 100 d (Tris buffer)	% deamidation after 100 d (phosphate buffer)
Gly	38	87.3
Ser	9.8	34.6
His	7.7	33.2
Ala	5.6	17.9
Asp	4.8	19.0
Glu	3.0	13.1
Asn	1.5	13.5
Thr	2.0	12.3
Lys	1.8	10.4
Gln	1.7	10.1
Cys	1.1	7.3
Lys	1.8	10.4
Gln	1.7	10.1
Arg	1.6	9.4
Phe	1.1	7.3
Met	0.9	5.4
Tyr	0.9	5.4
Trp	0.4	5.0
Leu	0.3	5.4
Val		2.8
Ile		1.3

Effect of Higher Order Structure on Asn Deamidation

In 1989, the ability of higher order structure to influence deamidation rates was just starting to be appreciated. In 1988, Kossiakoff demonstrated that polypeptide chain flexibility impacted deamidation rates (30). Other studies have since arrived at the same conclusions, examining the relative deamidation rates for Asn residues dispersed across a given globular protein structure (31,32). In addition, a number of studies have shown that placement of the reactive Asn residue within an ordered secondary structure slows the reaction rate. This has been found for α-helices (33,34), β-sheets (35), and β-turns (36,37).

Combining information about primary sequence along with the location of an Asn residue within a three-dimensional structure leads to improved predictive accuracy for deamidation rates (22). Moreover, alterations in the three-dimensional structure can affect deamidation rates. For example, addition of ligands that induce α-helical structure in insulin slow deamidation at Asn^{B3} (38).

Deamidation in Monoclonal Antibodies (MAbs)

Our knowledge regarding the stability and structure of MAbs has increased exponentially over the past 20 years. This includes detailed studies of deamidation in these pharmaceutically important molecules. In general, deamidation is responsible for much of the heterogeneity observed in MAbs along with other kinds of chemical instability and glycosylation differences (39).

In 1992, Kroon *et al.* reported that OKT-3, the first marketed monoclonal antibody product, undergoes deamidation (40). Subsequently, there were sporadic reports of deamidation in MAbs over the next decade (41–43). In the last 5 years, the number of reports on deamidation in MAbs has increased significantly. Some focus on the effect of extrinsic factors (44), some on sequence effects (26), while others emphasize the analytical methods used to monitor and quantify deamidation, which is primarily done by some type of mass spectrometry (26,45–57). These studies provide a solid basis for monitoring and quantifying deamidation in any protein or peptide. Other groups have reported using charge separation methods to detect and quantify deamidation in peptides and proteins (58–61), while others have employed RP HPLC (62,63), peptide mapping (64), and even Raman spectroscopy, which was reported to detect deamidation (65). However, the latter is quite insensitive, requiring deamidation to exceed 10%.

Prolonged storage of a human MAb resulted in deamidation at both Asn and Gln residues, as well as other chemical instabilities, such as fragmentation and pGlu formation (66–68). Those other degradation pathways are discussed below. What appears to be true is that the factors controlling deamidation rate (primary sequence, temperature, pH, *etc.*) in peptides and smaller proteins are equally important in MAb degradation.

Deamidation of Other Protein Pharmaceuticals

In addition to the large amount of work on MAbs, a number of other studies have appeared describing deamida-

Table III. Protein of Pharmaceutical Interst Where Deamidation has Been Observed

Compound	Reference
tPA	(69)
IL-11	(47,70)
rhGH	(71)
hGH	(72)
Tetanus vaccine	(73)
Anthrax vaccine	(74)
Anthrax protective antigen	(75)
Fc fusion protein	(76,77)
Glucagon	(78)
Lymphotoxin	(31)
Protein G	(79)
Hemoglobin	(80)
NGF	(81)
Hirudin	(82,83)
IL-1 receptor (type II)	(84)

tion in peptides and protein of pharmaceutical relevance, including vaccines and antigens (47,69–84). These include the entries in Table III. In general, any protein or peptide that contains one of these reactive Asn–Xaa sequences will be prone to deamidation over time.

Control of Deamidation Rates

A number of formulation approaches have been described to slow deamidation. The most effective approach is to control the pH. Deamidation for a single reactive Asn displays a V-shaped pH-rate profile, with the minimum being between pH 3 and 6. In addition, being a chemical reaction, it displays typical Arrhenius behavior, provided the protein or peptide does not change conformation appreciably across the temperature range of study.

Interestingly, it is possible to slow deamidation rates by altering the conformation of the protein. Even in 1989, it was known that a specific set of phi/psi angles is needed to allow the intramolecular nucleophilic attack to form the Asu intermediate (85). Phi and psi refer to the dihedral angles for the Cα–N bond and the C(O)-Cα bond, respectively. Therefore, limiting the flexibility of the peptide chain should and does slow deamidation. This is the basis for slower deamidation rates in well-defined and rigid higher-order structures (see above). It is possible to alter polypeptide chain flexibility using excluded solutes. Addition of sucrose to a flexible peptide caused it to adopt a β-turn conformation, thereby slowing deamidation (86). Sugars and polyols compact the structure of alcohol dehydrogenase and thereby slow deamidation in both the apo and holoenzymes (87). Similarly, removal of C-terminal amino acids in histidine-containing protein allows deamidation to proceed, presumably by removing steric constraints (88).

Finally, one can imagine that formulations that lower NH acidity would slow deamidation rates. This has been done using nonaqueous solvents (33,89), although these same solvents can also affect conformation, viscosity, and solvent dielectric, so the effect might not be entirely due to modulation of acid-base properties. The effect of viscosity has been described for model peptides (90,91). Similarly, dielectric and viscosity effects have been examined for Asp isomerization in MAbs (92,93). In that case, increased chemical stability was obtained at the expense of reduced conformational stability. Therefore, such approaches using nonaqueous solvents may not be viable for many globular proteins but could work for peptides, where solution conformation is less important to maintaining biological activity.

Prior to 1989, it was known that certain buffers exhibited buffer catalysis of Asn deamidation. Most buffers had been shown to exhibit some degree of buffer catalysis. Therefore, limiting the amount of buffer used should slow deamidation rates. In the last 20 years, relatively little has been done on this topic. Tyler-Cross and Schirch (29) demonstrated that deamidation of model peptides exhibited general base catalysis, but they did not observe specific base catalysis in their studies. So, apart from some observations on buffer effects, little has been done on mechanistic aspects of catalysis of deamidation. As for more recent observation on buffer effects, Girardet *et al.* reported that phosphate buffer increased deamidation rates in α-lactalbumin faster than tris buffer at pH 7.4 (94). Zheng and Janis conducted a detailed study on buffer effects on deamidation in a MAb, looking at tartrate, citrate, succinate, and phosphate (44). They found that citrate was the best choice, while the pH had to be less than 5.

Deamidation in the Solid State

The propensity of peptides and proteins to degrade chemically while in the solid state has been reviewed by Lai and Topp (95). Briefly, many of the reactions described here, including deamidation, have been observed for polypeptides in the solid state as well. For example, the deamidation rates of both cyclic and linear peptides were investigated in the solid state (37). A comparison of deamidation rates between solution and in the solid state can be found as well (96). Finally, Houchin and Topp (97) have recently reviewed the chemical degradation of peptides and proteins, including deamidation, encapsulated within PLGA microspheres.

Gln Deamidation

Our knowledge base regarding deamidation of Gln has increased tremendously over the past 20 years. It is still true that deamidation of Gln residues is less common than for Asn. Recall that cyclization of Asn residues leads to a five-membered ring. With Gln, that same intermediate is a six-membered ring, which is less favorable thermodynamically than the smaller ring. Certainly, Gln deamidation was known in 1989 (19). Yet, so little was reported that it was not discussed in our previous review. Since then, Joshi and Kirsch have reported some detailed mechanistic studies on Gln deamidation in peptides (78,98,99). A number of reports have found Gln deamidation in larger proteins, such as crystallins (100) and MAbs (101).

Theoetical Studies on Deamidation

In addition to the explosion of experimental studies on deamidation in peptides and proteins, a number of theoretical studies have emerged as well. These include molecular dynamics (MD) simulations (102) and *ab initio* calculations (103–105). Of note, Radkiewicz *et al.* 2001 showed that backbone conformation (i.e., phi–psi angles) affect acidity of the NH group (106). Gly, being able to sample more conformational space, shows

increased NH acidity, which would contribute to increased Asn deamidation rates. Therefore, the increased reactivity of Asn–Gly sequences might not be entirely due to lack of steric hindrance to intramolecular nucleophilic attack.

Succinimide Formation

In general, deamidated forms (Asp and isoAsp), as well as the corresponding cyclic imide (Asu) intermediates, have been isolated and identified, especially in peptides. The cyclic imide intermediate has been repeatedly isolated and characterized in monoclonal antibodies. Groups from Amgen used hydrophobic interaction chromatography (HIC), cation exchange chromatographjy (CEX), and liquid chromatography-mass spectrometry (LC–MS) to identify Asu formation in MAbs, especially IgG2s, that were stored at elevated temperatures (56,57,107,108). The primary degradation product appears to be the cyclic imide (Asu) intermediate at position 30 of the light chain (LC). Other studies have reported Asu formation at position LC32 (109) and residue 102 of the heavy chain (43).

Succinimide formation has been reported in other systems. For example, stressed samples of hGH form a succinimide product at an Asp–Gly site that was isolated and quantified using reversed-phase HPLC (110). Similar degradation has been reported for glial cell line-derived neurotrophic factor, which forms a succinimide product at position 96 (111). The degraded form was identical to the native protein in structure, pharmacokinetics and activity. Lysozyme has also been reported to form a succinimide product at a Asp–Gly site as well (112).

Asp Isomerization

Once the cyclic imide intermediate forms, it can open to form either Asp or isoAsp products (Fig. 1). Such a mechanism indicates that Asp itself could cyclize to form the same succinimide (Asu) species, thereby allowing conversion from Asp to isoAsp. This reaction has been called Asp–isoAsp interconversion, but is more commonly referred to as Asp isomerization. The rate-limiting step is the same for both deamidation and Asp isomerization, that is, the rate is controlled by formation of the cyclic imide intermediate. Consequently, the same approach can be taken to slow each reaction. In other words, pH provides the greatest degree of control by slowing deprotonation that leads to intramolecular cyclization. Early work on this reaction indicated that only the protonated form of Asp isomerizes, i.e., there is much lower reaction rate above pH 5 (113). In fact, above pH 8, the reaction is independent of pH and buffer concentration. Below pH 3, only hydrolysis is observed. The size of the C-terminal amino acid retards the formation of the cyclic imide intermediate (114), thereby slowing Asp isomerization. Steric constraints affect cyclization rates, as with deamidation (88).

Since the original review was published, Asp isomerization has been reported in many systems, especially monoclonal antibodies (27,43,55,92,93,107,108,115). Some of the same LC-MS methods used to identify deamidation in MAbs have been used to monitor Asp isomerization as well (27,55,116). Both degradation pathways have been observed in MAbs (43,55). Specifically, Asp isomerization has been reported at position 32 in the light chain (93,109) and position 102 in the heavy chain (43). For Asp–Asp sequences in MAbs, both Asp isomerization and Asp-assisted hydrolysis were observed (27).

Racemization (which is discussed in more detail below) has been observed concomitantly with Asp isomerization (63), similar to the observations with deamidation (13,15). This emphasizes once again how interconnected many of these chemical degradation pathways can be.

Other proteins of pharmaceutical interest besides MAbs have been reported to undergo Asp isomerization. For example, Asp^{93} isomerization has been shown to be the primary degradation pathway for NGF (81), while Asp isomerization (at Asp^{45} and Asp^{47}) has been found in IL-11 as well (70). Dette and Wätzig were able to resolve the isoAsp product of Asp isomerization in recombinant hirudin using capillary electrophoresis (117).

Outside of controlling pH and temperature (see above), little has been reported on formulation strategies to slow Asp isomerization. The use of excluded solutes to provide conformational stability in a MAb actually decreased chemical stability by accelerating Asp isomerization (92). Presumably, changing the succeeding amino acid (in the n+1 position) would also slow the reaction, but no detailed studies of that type have been reported.

Asp Hydrolysis

There is a third reaction that is associated with degradation at As/Asp residues and that is Asp-associated hydrolysis of the peptide backbone (also known as proteolysis). Unfortunately, there are few reviews available on the topic, with the most extensive dating back to 1983 (118). Since this reaction also involves intramolecular cyclization, it is not surprising that proteolysis shows the same pH-rate profile and sensitivity to buffer catalysis as deamidation (119). The mechanism was delineated in detail by Joshi and Kirsch (78), with nucleophilic attack occurring at the ionized side chain of Asp on the protonated carbonyl of the peptide backbone. This produces an anhydride species and release of the N-terminal portion of the peptide chain. There is some information available on the effect of primary structure on Asp hydrolysis. The presence of Ser or Tyr at position n+1 can accelerate reaction (98,99). Similarly, having Ser or Val at position n+1 accelerates hydrolysis relative to Asp isomerization (114).

Other similar hydrolysis reactions have been reported. For example, the Asn–Pro bond appears to be particularly labile in the presence of ammonia (120). A similar degradation process has been reported for the Asp^{60}–Pro^{61} bond in NGF (81). The peptide linkages in either side of Pro and Trp were found to hydrolyze in spantide II, a bioactive peptide (121).

Hinge Region Hydrolysis

Hydrolysis of the peptide backbone has been seen in antibodies even when Asp is not present. This reaction occurs most frequently within the hinge region of the antibody, so it is known as hinge region hydrolysis. However, it can occur at the C_H2–C_H3 interface as well (67). Typically, it occurs in IgG1s, so the reaction is likely influenced by the flexibility of the peptide chain. This reaction is distinct from the enzymatic hydrolysis that can occur in this region with antibodies (39).

There have been a number of detailed studies on this process. The first study reported cleavage in the hinge region of mouse MAbs (122), showing that the reaction can occur under basic pH conditions (122). Fragmentation, along with other chemical instabilities, was reported in OKT3, which is a mouse IgG2a antibody (40,123). Using MALDI-TOF and capillary electrophoresis, Alexander and Hughes found hinge region hydrolysis to occur in chimeric mouse/human IgGs (124), as was also reported by Paborji *et al.* (125).

The general nature of this reaction was shown by Cordoba *et al.*, who showed that hinge region hydrolysis occurred in four different human IgG1s (126). The observed fragmentation pattern indicated that the hydrolysis reaction is not specific to a particular peptide bond, but occurs within a narrow range of residues. In this case, hydrolysis was limited to the heavy chain sequence Ser-Cys-Asp-Lys-Thr-His-Thr. Similarly, descriptions of hinge region hydrolysis, detected in the course of mass spectrometry studies on MAbs, were reported as well (127,128). While chain flexibility appears to be important, recently it was demonstrated that conformational instability of Fab region leads to increased rates of hinge region hydrolysis as well (129).

The pH-rate profile for hinge region hydrolysis is V-shaped (130), with a minimum near pH 6. The rate increases linearly with pH above pH 6. The study by Cordoba *et al.* indicated that EDTA and protease inhibitors have no effect on hydrolysis rates (126). In addition to the more general hinge region hydrolysis described above, there have been reports of metal-assisted hydrolysis of MAbs in the same region (131,132). In these cases, chelating agents have some ability to slow degradation.

Trp Hydrolysis

In addition to these better-known degradation processes, other functional groups are also sensitive to hydrolysis. For example, the side chain of Trp is known to undergo hydrolysis. The primary degradation product is called kynurenine (133–135), which fluoresces at much longer wavelength (450 nm) than Trp itself. Kynurenine and related substances can also form during oxidative degradation of Trp as well (see below).

Racemization and β-Elimination

These two degradation pathways are interrelated, as the initial step is the same: deprotonation of the hydrogen on the α-carbon (Fig. 2). Usually, C–H bonds have little acid-base reactivity, but the C–H bond of an amino acid does have some acidic character. As a result, racemization is usually a very slow process, so slow that it can be used to date artifacts. *In vivo*, a number of proteins have been reported to racemize, as in crystallins from the lens of the eye (136,137) and myelin in muscle (138).

Typically, the racemization occurs at Asp residues (138), although racemization at Asn^{127} in murine lysozyme has been reported (139). Why this residue is more reactive is not yet known. A more extensive summary of amino acid racemization can be found in the review by McCudden and Kraus (140).

Once the Cα–H bond ionizes, recombination can lead to racemization (Fig. 2). On the other hand, the resulting carbanion can rearrange and eject a leaving group from the β-carbon, producing a double bond between the alpha- and beta-carbon. This is β-elimination. At high temperatures, it appears that β-elimination of Cys residues occurs readily in a number of proteins (141). Among proteins of pharmaceutical interest, β-elimination has been reported for IL-1ra (142) and insulin (143). It has also been shown that β-elimination occurs under conditions causing hinge region hydrolysis (144).

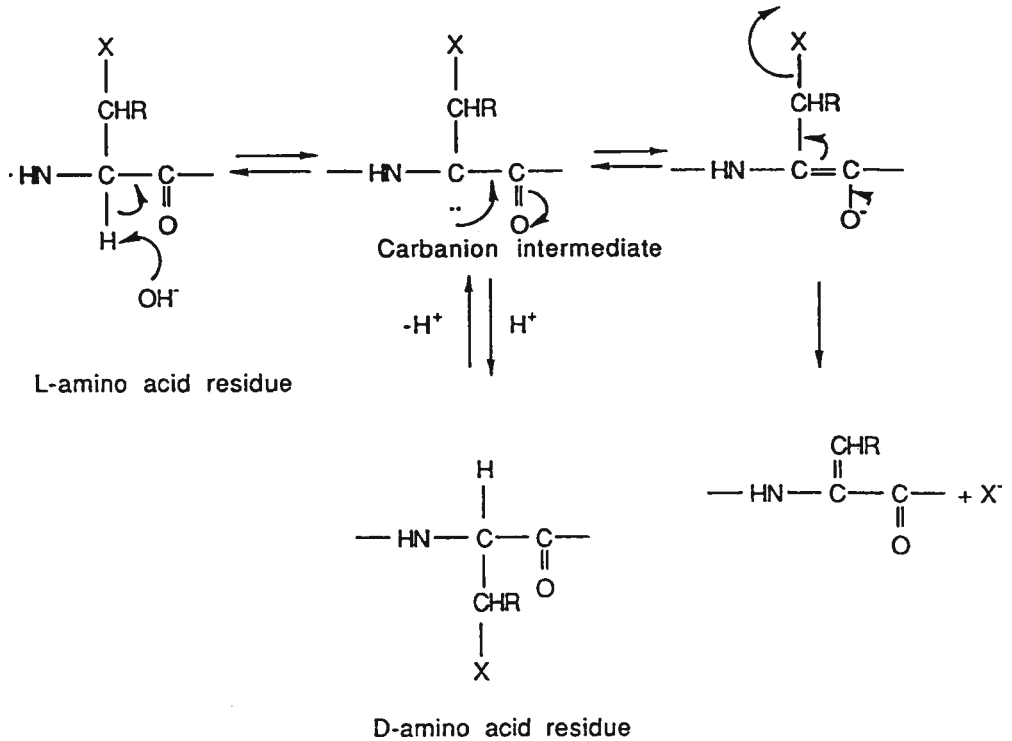

Fig. 2. General mechanism for racemization and β-elimination in proteins (taken from reference 1).

DKP Formation

One other N-terminal cyclization process has been described in some detail. Note that the N-terminal amino group can be a potent nucleophile, especially above pH 8. If the amine attacks the second carbonyl group in the peptide backbone, a diketopiperazine (DKP) ring is formed. Degradation of the N-terminus of a peptide or protein by DKP formation has been commonly observed during long-term storage and during peptide synthesis (145–147).

This reaction was initially observed in peptides (148), where the DKP ring can rearrange, either with loss of the first two amino acids or reversal of their positions in the chain. The extent of DKP formation depends on percentage of terminal amino groups in the free base form (17,149,150). Under acidic conditions, the reaction is quite slow and pH-independent. Kinetic analyses of DKP formation in peptides have examined the effects of pH, buffer type and concentration and temperature (17,149–151). The first-order rate constant generally increases with increasing buffer concentration, except for carbonate, which shows no concentration dependence (150). Degradation caused by DKP formation was shown to be responsible for the N-terminal heterogeneity observed in hGH (145) and substance P (152). Further details of the reaction kinetics of DKP formation have been presented recently (17).

To the extent that DKP formation leads to reduction in the length of the polypeptide chain, it can be considered a proteolytic reaction. Rearrangement of a DKP from the first two amino acids, via cleavage of the peptide bond C-terminal to the second amino acid, produces a clipped protein reduced in molecular weight by the mass of the two amino acids. In solution, DKP formation is common for proteins with the N-terminal sequence NH_2-Gly-Pro (153).

pGlu Formation

This reaction was not covered in the original 1989 review, although there were some literature references prior to that date (e.g., references 154–156). This reaction involves nucleophilic attack of the N-terminal amine on the side chain of a Glu residue (and occasionally a Gln residue) to form a five-membered ring structure (Fig. 3). In other words, non-enzymatic formation of pyroGlu (pGlu) follows a mechanism similar to that for DKP formation in that it involves nucleophilic attack of the N-terminal amine on the polypeptide chain. In this case, site of attack is the carbonyl group of the N-terminal Glu side chain, resulting in elimination of water (Fig. 3). This cyclized, N-terminal structure is often observed in monoclonal antibodies due to the frequency of Glu in the first position of the light chain and occasionally in the heavy chain (45,67,157–162). Most often, the presence of pGlu is detected using mass spectrometric techniques. The pGlu degradation product has been seen to increase during prolonged storage of MAbs (67,160). In some cases, the conversion to pGlu at the N-terminus of the heavy chain has been quantitative (127). Formation of pGlu has also been reported in variants of BMP-15 (163).

As the reaction involves nucleophilic attack, rates of pGlu formation are typically pH-dependent. The pH dependence of the reaction has been reported (67,162), although the data are quite limited compared to the detailed pH profiles published for other hydrolytic reactions. It appears that the nature of the buffer has an effect on the rate of pGlu formation (162,164). Phosphate appears to cause more rapid cyclization, at least in model peptides (164). At lower pH, acetate appears to be the best buffer species for slowing pGlu formation (162). Finally, it has been reported that pGlu can be formed from N-terminal Gln residues as well as Glu, although the reaction appears to be slower with Gln than with Glu (154,165).

Glycation of Proteins

Glycation occurs when a protein is incubated in the presence of a reducing sugar. It involves the reaction with a base, typically the side chain of lysine and a carbonyl group of a reducing sugar. This leads to formation of a Schiff base, which can undergo rearrangement to more stable products. Altogether, these associated reactions are termed the Maillard reaction or non-enzymatic browning after the color that evolves.

The Maillard reaction can occur in the solid state as well as in aqueous solution (95). For example, recombinant DNAse I undergoes glycation in dried formulations (166). Glycation occurs *in vivo* as well as *in vitro*. In fact, the extent of glycation of hemoglobin *in vivo* is a distinctive marker for diabetes (167).

The mechanism of glycation has been outlined in some detail recently (168). When glycation occurs, it is known that it can affect function (169), although it has been shown not to affect binding affinity of certain antibodies (168). However, it can affect the overall stability of the molecule. For example, the Maillard reaction can lead to more labile peptide linkage, as in relaxin (170).

This degradation pathway is one of the primary reasons that formulation scientists tend to avoid using reducing sugars (glucose, lactose, fructose, maltose) in formulations. However, reducing sugars can be generated *in situ* by hydrolysis of sucrose. Smales *et al.* demonstrated this for sucrose-based formulations undergoing viral inactivation at elevated temperatures (171,172). Similarly, glycation in sucrose-based formulations has been observed during storage studies as

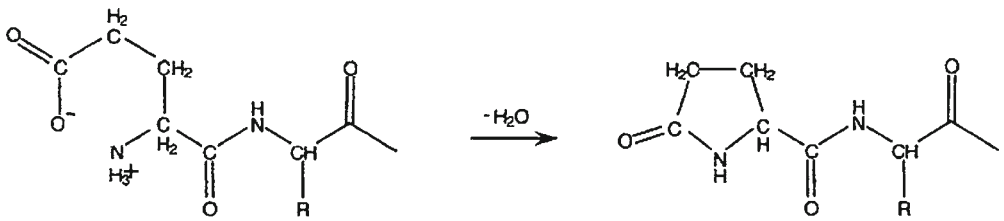

Fig. 3. Conversion of Glu to pGlu.

well (173–175). However, these usually require elevated temperatures and acidic pH. Note that the glycosidic bond of trehalose appears to be much stronger than for sucrose, as trehalose formulations rarely, if ever, display this type of decomposition. On the other hand, low pH has been shown to lead to sucrose instability and subsequent glycation (174). In fact, glycation happens about 2,000-fold faster with sucrose than trehalose at pH 2.5 (176) due to formation of glucose and fructose upon sucrose hydrolysis.

The sites of glycation usually involve lysine residues, although Arg residues and the N-terminus can be involved as well. It is known that certain lysines are more reactive in MAbs (177) than others with respect to glycation. The basis for the enhanced reactivity is unclear, although basicity and solvent accessibility are likely to be important. Glycation has been seen for IgG2s (178), as well as IgG1s (177). Some buffer catalysis has been observed for glycation (179), at least with γ-globulin, with phosphate accelerating the reaction. However, buffer catalysis was not seen with BSA or ovalbumin.

Oxidation

Chemical degradation of a protein due to oxidation is the other primary degradation process that occurs, along with the hydrolytic reactions described above. Any protein that contains His, Met, Cys, Tyr and Trp amino acids can be potentially damaged by reaction with any of a number of reactive oxygen species (ROS) (180–184). Oxidation of these reactive side chains in a protein can occur during any stage of protein production, purification, formulation and storage (40,185–187).

Our understanding of chemical degradation caused by oxidation of proteins has greatly expanded over the last 20 years. The rate of oxidation is affected by both intrinsic and extrinsic factors. Intrinsic actors include the flexibility of the peptide backbone (188) and the overall structure of the protein (188–190). In addition, extrinsic factors, such as pH (191–193) and buffer type (191,194), can affect oxidation rates of proteins as well.

Oxidation of proteins and peptides are usually broken down into two general categories: site-specific (i.e., metal catalyzed oxidation or MCO) and non-site specific reactions, which includes photooxidation and free radical cascades. The latter can be initiated from a variety of sources, leading primarily to Met and Trp oxidation.

Met Oxidation

The chemical stability of methionine (Met) residues has been shown to be important for conformational stability and protein function (191,195–197). Unlike hydrolysis reactions, it appears that Met oxidation is nearly pH-independent (191). Therefore, one cannot control oxidation by adjusting pH. Oxidation of Met can be accomplished with a wide range of ROS. Even molecular oxygen is potent enough to convert the side chain of Met to its corresponding sulfoxide. One must keep in mind that reaction with oxygen in aqueous solution will involve the temperature dependence of oxygen solubility in water, which increases as the temperature is lowered (198). So, while Met oxidation typically follows Arrhenius behavior, reactivity may actually increase in refrigerated samples due to the higher solubility of oxygen than at room temperature. As these reactions are propagated by free radicals, it must be recognized that there are many potential sources of free radicals, including containers (199,200) and excipients (see below).

Even at the time of the 1989 review, it was known that different Met residues oxidized at different rates (201). However, little was known beyond that observation. What appears to be most important in controlling oxidation rates in proteins is the degree of solvent accessibility of the particular residue, allowing the oxidizing species to attack the side chain readily. Therefore, a Met residue that is fully solvent-exposed will exhibit a maximal rate of oxidation, while a buried side chain will oxidize very slowly (187,202–205). In other words, the ROS must be able to access the side chain easily for oxidation to proceed rapidly. This also means that while proteins have some ability to protect certain groups against Met oxidation because of their ability to bury the side chain in the interior of the protein, peptides do not have this ability to protect themselves against oxidation (206). Peptides lack higher order structures, causing the amino acids to be fully solvent-exposed at all times, resulting in maximum rate of oxidation for Met.

So, while solvent accessibility is key for controlling oxidation rates, it may not be the only important factor. Some evidence has also been presented that the rate of Met oxidation is linked to or correlated with conformational stability (207–209). Moreover, near the melting temperature of the protein, one can observe non-Arrhenius kinetics due to large-scale structural changes (208).

Oxidation of Met residues has been widely reported for MAbs, especially those in the Fc region (186,202,210). In one study, the exact distribution of oxidized residues differs whether the protein is stored for an extended period of time or subjected to t-BuOOH (186). This illustrates the fact that, while forced oxidation studies are valuable in formulation screening, they may not produce the exact distribution of products seen during long-term storage.

Metal-Catalyzed Oxidation (MCO)

Metal-catalyzed oxidation occurs when a redox active metal binds to a protein. The ligands are often Gly, Asp, His and Cys. Of these amino acids, His and Cys are sensitive to oxidative damage, as the ROS generated at the metal center does not have to diffuse very far before reacting with the protein (211). Mechanistic studies show that the metal ion and peroxides undergo a Fenton-type reaction, creating free radicals (212). The products of the oxidation of His are varied (213), but 2-oxo-His appears to be the major oxidation product. The 2-oxo-His product has been detected in human relaxin (214), prolastin (215) and human growth hormone (211,216).

Trp Oxidation

Oxidation of Trp residue can occur, even in the absence of light. The primary products are kynurenine derivatives (217), especially when iron-based oxidants are used. The oxidation of Trp has been seen in MAbs, leading to new peaks in both SEC and RP HPLC chromatograms (218).

Photooxidation

In 1989, little was known about photolytic degradation of proteins beyond some reports regarding heme proteins that

absorbed in the visible wavelength region. Since then, exposure to light has been recognized as a potential source of chemical degradation, as reflected in the ICH Guidelines Q1B. Recently, a number of reviews have been published that describe sources of light exposure, degradation mechanisms and potential methods to reduce damage caused by light (183,185). When a protein is exposed to light, species are generated that will cause chemical oxidation to amino acids that are sensitive to the light-induced oxidation, which are Trp, Tyr and Phe. The light-induced oxidation reaction pathway starts when a photon is absorbed, causing an electron to enter an excited state (185). From the excited state, the amino acid has a number of degradation pathways resulting in different products. It now appears that photodegradation, especially photooxidation, is a common degradation pathway for many proteins, as exemplified by photodegradation on numerous proteins found in milk (219).

Trp is most sensitive to the light above 300 nm of the four amino acids, as it absorbs the bulk of the light at these wavelengths. Recently, a number of examples of photolytic damage to pharmaceutical proteins have been reported, primarily involving photooxidation of Trp. In IgG monoclonal antibodies, the oxidation of the Trp appears to cause loss of bioactivity and discoloration for high concentration formulations (192). The rate of oxidation for the high concentration antibody formulation is a pH-dependent process and, at basic conditions, can result in soluble aggregates (192). Another MAb, MEDI-493, showed a loss of binding and biological activity when irradiated with UV light, caused by oxidation of Trp (220). Another study measured the effect of exposing three different monoclonal antibodies to 254 nm; all three antibodies showed an increase in the percentage of aggregates over the course of the study (221). Structural changes were observed in recombinant human interferon-α_{2a} when exposed to UV radiation measured by absorbance, circular dichroism (CD) and fluorescence (222).

When Trp is photoexcited by absorbing near-UV light, it can affect neighboring amino acids and, in doing so, can reduce disulfide bonds (193,223). The photoionization of Trp can reduce disulfide bonds by electron transfer, resulting in chemical and physical degradation of the protein (193,223–225). Studies with different proteins have demonstrated the ability of photoexcited Trp to reduce disulfide bonds in both the liquid (223,224,226,227) and solid-state matrixes (225).

Please consider one final note on photooxidation. It is possible for photooxidation to be facilitated by polysorbate, that is, polysorbates (also known by their trade name as Tweens®) have been shown to be photoenhancers, leading to more facile production of singlet oxygen (228). Therefore, increased oxidation of proteins may not be solely due to oxidative impurities in the surfactant.

Cysteine Oxidation

While the primary oxidative process involving Cys residues is formation of disulfide linkages (see below), they are also subject to other oxidation processes as well. For example, they can form sulfenyl species (229), where an oxygen atom is added, in much the same way that Met is oxidized to a sulfoxide. Thiol oxidation has been shown to ablate the activity of alcohol dehydrogenase (87). In addition, there is a growing body of literature on formation of thiyl species, sulfur-based free radicals that can form from photolytic initiation or disulfide decomposition (230).

Protection Against Oxidation

A number of approaches can be used to limit oxidation of the proteins, which are dependent oxidation mechanism. Oxidative damage caused when a protein is exposed to UV radiation can be limited by adjusting the secondary packaging and addition of additives to the formulation (185,192). Otherwise, formulation strategies for mitigating oxidation are somewhat limited. Minimizing exposure to oxygen, by reducing the headspace in the vial, appears to be effective (225,228,231). This is particularly important for Met oxidation, where even less potent oxidants can cause damage.

Limiting the solvent accessibility of oxidation-sensitive side chains is one possible strategy, which has been shown to work for subtilisin (232) and alkaline protease (233). On the other hand, sucrose increases the oxidation rate of Factor VII (234), for reasons that are not yet known. While compounds, such as mannitol, have been reported to be free radical scavengers (235,236), there are no reports of them being effective at reducing oxidation in biopharmaceutical proteins.

However, it has been shown that sugars and polyols can complex metals at high concentrations, thereby reducing MCO-induced damage (237). Sacrificial additives can be employed, with the intention of having these compounds oxidized instead of the active ingredient. This has been shown to be an effective strategy for free Met (228,238,239), for N-Ac-Met (240), for thiosulfate (228), and for N-Ac-Trp (241).

Control of MCO can be accomplished, in some cases, by the addition of EDTA (242,243). Such chelating agents can reduce the reactivity of these metals. Keep in mind that the binding affinity of EDTA decreases significantly below pH 5, where the carboxylate side chains become protonated. Note that antioxidants, such as ascorbate, while effective at reducing lipid peroxidation, can actually increase the reactivity of transition metals and increase MCO-mediated damage (211,244–246). Otherwise, minimizing the levels in the bulk drug and excipients used is the other factor that will lead to improved storage stability with respect to oxidation. Many excipients carry oxidative impurities, including polysorbates (247–249) and PEG (242,250,251). A review of peroxide impurities in excipients has been published (252).

Disulfide Scrambling

The ability of Cys residues to form disulfide bonds has been known for decades. While they can play a significant role in aggregation, through covalent cross-linking (see below), they can also affect the overall conformation of a protein, as occurs during rearrangement of existing disulfides within a molecule. As discussed above, removal of free Cys residues (the reduced form), which can act as the starting point for disulfide scrambling or exchange, can retard this process significantly. Even though the number and type of chemical bonds in the rearranged forms are identical, because chemical bonds have been broken and formed, this should be considered a type of chemical instability.

A number of articles have now appeared about the formation of disulfide isoforms in IgG2 monoclonal anti-

bodies. The preferred method for detecting and quantifying these disulfide species appears to be capillary electrophoresis (CE) in the presence of SDS (253–255), although RP-HPLC and LC-MS has also been reported to resolve them as well (128). There appears to be a functional difference associated with placement of the disulfides in IgG2s (256,257). This demonstrates how rapidly our understanding of the molecular details of these complex molecules is evolving.

PHYSICAL INSTABILITY

Physical instability refers to any process whereby the protein changes its physical state without any change in the chemical composition. In particular, this review, like the one in 1989, will focus on four processes: denaturation, surface adsorption, aggregation and precipitation. For the purposes of this review, aggregation is restricted to formation of soluble aggregates, where precipitation refers to a macroscopic event where the protein can be seen coming out of solution. As seen below, precipitation may or may not be connected with aggregation. It may simply be due to conditions whereby the protein has exceeded its solubility limit.

Each of these four topics is extensive and continues to evolve. Therefore, the purpose is not to provide a comprehensive overview of each topic. Rather, the intent is to demonstrate how our understanding of protein stability in each of these areas has increased over the past 20 years or so.

Denaturation

Denaturation denotes the loss of the globular or three-dimensional structure that most proteins adopt. This globular structure is referred to as the native state, although it is well understood that it is really a multiplicity of microstates (258). Consequently, upon unfolding or denaturation, the protein changes its physical state, but the chemical composition remains the same. Denaturation can involve the loss of secondary or tertiary structure (or both).

Thermal Denaturation

Probably the most common stress that causes the globular structure of proteins to be lost is elevated temperature. A plot of temperature *vs*. the fraction of unfolded protein is sigmoidal, with the midpoint being denoted as the T_m value (standing for the temperature of melting). In general, one can imagine that increasing T_m reflects an increase in conformational stability. Assuming that the thermal transition from the folded to the unfolded state has a similar degree of reversibility (see below), this is possibly true (259). However, in the past 20 years, it has been shown that reversibility may be an even better indicator of storage stability than T_m values (260,261). Consequently, other measures of conformational stability, as from chemical denaturation studies (see below), may be more reliable for guiding formulation decisions.

Most often, thermally induced denaturation is irreversible, as the unfolded protein molecules rapidly associate to form aggregates. This behavior is often observed during thermal denaturation studies using DSC. Even since the report by Sanchez-Ruiz *et al*. on using the scan rate dependence of T_m (262), there have been numerous reports of using DSC to examine aggregation rates by varying the scan rate. The problem has been that it assumes certain kinetics. More recently, efforts have been made to develop more general kinetic schemes (e.g., references 263,264). While they remove some of the limitations of previous approaches regarding the reaction order, the mathematics associated with these schemes can be quite involved.

Cold Denaturation

While the process of cold denaturation has been known since 1961 (265), there have been few reports of proteins undergoing cold denaturation (266). This is because the vast majority of proteins exhibit cold denaturation well below the freezing point of water. This would imply that it is not of great significance with respect to protein denaturation. However, one must realize that the glass transition temperature of the maximally freeze-concentrated state (T_g') is usually well below −20°C, even in the presence of common stabilizers, such as sugars. This means that proteins will have a mobility in the −20°C frozen state that is similar to that in fluid solution. Consequently, the potential for cold denaturation may be greater than previously believed. For example, a recent study on IL-1ra estimated that the cold denaturation temperature is ∼−10°C (209), easily accessible in the frozen state unless the storage temperature is well below −30°C.

Chemical Denaturation

Another common method used to unfold proteins, and thereby determine the free energy of unfolding, is the addition of chaotropes, that is, compounds that cause the loss of the globular structure of proteins. The most common of these, by far, are urea and guanidinium hydrochloride (GnHCl). Analysis of these sigmoidal curves to determine the free energy of unfolding (ΔG_u) has been summarized quite well in reviews by Pace and coworkers (267–269).

There is a difference of opinion as to whether the free energy of unfolding indicated by thermally induced denaturation and chemically induced denaturation correlate. For example, one group finds a good correlation (270), while one group finds just the opposite (271). The differences might be due to the variability in protein size, slight differences in reversibility or even differences in the temperature dependence of the pre- and post-transition regions (272).

The mechanism by which these compounds disrupt and destabilize the globular structure is still under intense investigation. Even in the past year, a number of papers have appeared about whether a chaotrope destabilizes the native state or stabilizes the unfolded state (273–276). In addition, urea appears to impede the hydrophobic collapse associated with formation of the globular native state (275). One thing is clear. Unlike excluded solutes, chaotropes appear to bind to proteins, reducing their chemical potential. As the unfolded state has a much larger surface area than the native state (usually), the chemical potential of the unfolded state is lowered to a greater degree. When it falls below that of the native state, the protein unfolds. It has been reported that the addition of high concentrations of either urea or GnHCl can alter the pKa of amino acid side chains by 0.3 to 0.5 units

(277). This alone could affect the conformational stability of the protein via increased electrostatic repulsion.

Pressure-Induced Denaturation

Another area that was virtually unknown in 1989 is the idea of using high pressure to unfold proteins. Since then, a number of good articles on the topic have appeared (278,279). Typically, pressures of greater than 2,000 bar (~2,000 atmospheres) are required, with up to 4,000 bar often needed (279). The molecular basis for pressure-induced denaturation was recently described (280). Also, the ability of osmolytes or excluded solutes to stabilize proteins appears to work for pressure-induced denaturation as well (281). In general, pressure-induced denaturation appears to be fully reversible, unlike other stresses that cause protein unfolding (e.g., reference 282). It should be noted that intermediate pressures, 1,000–1,500 bar, can be used to dissociate aggregates and allow for facile refolding of aggregated protein (283,284).

Denaturation in the Solid State

Proteins can denature at elevated temperatures, even when in the solid state, where one would imagine mobility is quite limited. For the most part, the reported denaturation temperatures for dried proteins are quite high, often above 150°C (285–287). The T_m value, like the T_g value, appears to scale with moisture content and with each other (287–289). A detailed discussion of how freeze-dried proteins denature has recently been published (288,290), as it relates to T_g and other glassy state behavior. For example, for hGH, the denaturation only occurs above T_g, is cooperative and is mostly irreversible (290).

Intrinsically Denatured Proteins

Over the past decade, there has been a realization that many proteins exist, under native conditions, as unfolded structures (i.e., so-called random coils). The current designation for these proteins is intrinsically denatured proteins (IDPs). More than 50 such proteins have now been identified, and the topic has been recently reviewed (291–293). IDPs include some proteins of pharmaceutical interest, especially those of the acidic fibroblast growth factor superfamily. Therefore, proteins can be functional without having a globular fold. In these systems, denaturation, in the usually sense, does not apply.

Aggregation

Since the 1989 review appeared, the subject of protein aggregation has become one of the most highly debated and researched areas in the field of protein stability. Consequently, there have been a large number of studies on the topic, and many excellent reviews have been published (e.g., references 294–299). Therefore, only a brief overview will be given here. While the number and classification of general aggregation mechanisms varies from review to review, it appears that there are five general mechanisms (298), as summarized in Table IV.

Table IV. General Mechanisms of Protein Aggregation (as Described in Reference 298)

Mechanism	Description
1	Association of native monomers (as with assembly of insulin hexamers)
2	Aggregation of conformationally altered monomers (conformational change is the rate-limiting step)
3	Aggregation of chemically-modified monomers (may follow behavior of mechanisms 2 or 4)
4	Nucleation-controlled aggregation (involves formation of critical nucleus or seed)
5	Surface-induced aggregation

Aside from its role in the pathogenesis of amyloid diseases, protein aggregation is a frequently cited challenge in the manufacturing and development of protein therapeutics. Non-native aggregation has received much attention from industry, academia, as well as regulatory agencies in recent years for a number of reasons. First, aggregates of therapeutic proteins can increase the likelihood of adverse immunogenic effects during therapy, which has been linked to increased patient morbidity or mortality (300,301). Second, the biological function of the molecule can be compromised in non-native aggregates, thus reducing its efficacy. Finally, aggregated protein can make a solution appear turbid or physically separate from the solution, thus reducing its pharmaceutical elegance and making the drug product unacceptable for use by health care professionals.

Protein aggregation is a term that can include many types of molecular assemblies. Aggregation can arise from non-covalent interactions or from covalently linked species, which can vary widely in terms of reversibility (295–297). One of the greatest challenges to the investigation of the causes of protein aggregation is that there is no single pathway by which proteins can form an aggregate (Table IV). Aggregation can have many causes, such as incorrect folding during protein expression, and perturbation of the native conformation during protein purification, formulation, freeze-thawing, freeze-drying, ultrafiltration/diafiltration, vial and syringe filling, pumping, transportation or storage (302–306). These processes can jeopardize product stability by exposing proteins to potentially damaging conditions, such as freezing, dehydration, extreme pH, air–liquid interfaces, solid–liquid interfaces, or high or low temperatures.

Despite the diversity of potential causes and aggregation pathways that are in existence, current paradigm is that, in order to better control protein aggregation during processing and storage, it is important to consider the roles of intrinsic conformational stability of a protein as well as protein–protein interaction. In general, conformational stability has been considered the most influential factor in modulating aggregation. This is because nonnative aggregation of protein molecules starts from a partially unfolded state; thus, the level of transient reactive species, sometimes referred to as N* (due to its structural similarity to the native state), is thought to be the rate-limiting factor in protein aggregation (307–310). For example, aggregation of human growth hormone and acidic fibroblast growth factor has been effectively suppressed by additional of stabilizers that thermodynamically favor the native state (311,312). In these cases the stabilizing agents exert their

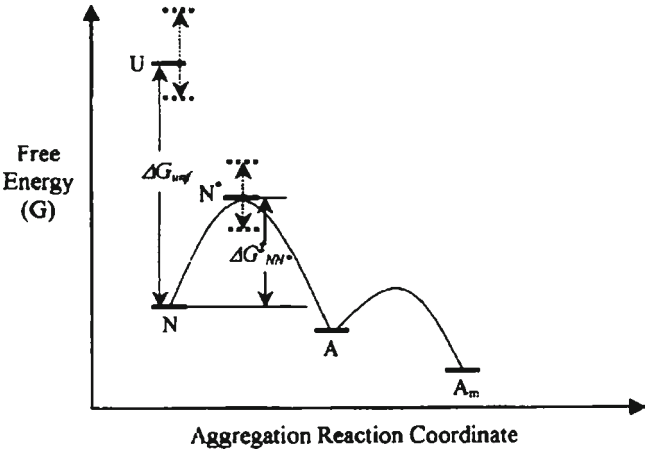

Fig. 4. Schematic reaction profile for aggregation of interferon-γ. A is the monomer aggregation intermediate. Dotted upward and downward arrows illustrate the shifts in free energy (relative to the native state, N) of the aggregation transition state, N*, and the unfolded state, U, when sucrose or GnHCl is added, respectively (taken from reference 296).

effect by preferentially binding to the native protein, thus reducing the equilibrium concentration of aggregation-competent species in solution. In another case, aggregation of recombinant human interferon-γ was inhibited by addition of sucrose, which increases the thermodynamic barrier between the native state and aggregation-prone state (Fig. 4). Similarly, the denaturant, guanidinium hydrochloride, lowers the barer, and aggregation rates increase. The stabilizing effect of sucrose occurs by way of the preferential exclusion mechanism pioneered by Timasheff and coworkers (308,313–315). In all of these cases, protein aggregation is reduced or inhibited under solution conditions that also increase the ΔG_u.

In order to understand the mechanism of aggregation and possibly design approaches to reduce aggregate formation, many measure aggregation kinetics. Some excellent overviews are available on the topic and make for excellent reading (296,316,317). The difficulty lies in that there are a multitude of possible kinetic schemes that can be envisioned (318). Distinguishing between them can be difficult, although a scheme proposed by Morris *et al.* appears to fit a number of data sets in the literature. In fact, many of the leading researchers in the field believe that unless elaborate kinetic studies are performed, it is nearly impossible to arrive at a unique mechanistic scheme (319,320). Therefore, it is expected that this area will continue to develop, as the need for fundamental understanding of the molecular details of aggregation is so great.

Precipitation/Particle Formation

The 1989 review listed precipitation as one of the four major physical instability pathways (1). It is important to specify what is meant by precipitation. On one hand, soluble aggregate formation can continue until the aggregates are so large that they can no longer remain soluble. This results in a macroscopic manifestation of aggregation that we observe as haziness or cloudiness. Often, this is now termed particle or particulate formation. This behavior is irreversible, and the protein is partially or completely unfolded. Formation of particulates has now become an important scientific and regulatory focus in the development of protein therapeutics.

There are compendial methods for measuring particulates in injectable products (321), such as USP method <788>. However, this method focuses only on particles above 10 and 25 μm. More recently, subvisible particulates have received a great deal of attention, both from regulatory agencies as well as researchers in the field (322). There is concern that these might be the most immunogenic of particulates found in protein products (301). Furthermore, new analytical methods, such as micro-flow imaging (MFI) allow one to not only quantify particles across this size range (323,324) but also capture images of the individual particles, making it possible to distinguish protein aggregates from foreign materials (325).

On the other hand, not all insoluble protein material is due to aggregation. One could have a protein that is salted-out, that is, the addition of an excluded solute has caused the chemical potential of the protein to exceed that of the solid phase (326,327). While our understanding of protein solubility is still imperfect, there have been significant advances in the past 20 years (326,328). Salted-out proteins still retain activity and native-like structure (327,329–331), and the precipitation is fully reversible upon dilution.

Surface Adsorption

Given the myriad of surfaces a protein may encounter during bioprocessing and in the final dosage form, interfacial stability is an important factor that cannot be underestimated. Adsorption itself is a physical instability, as it changes the physical state of the protein. However, even more problematic is the subsequent damage that can occur upon interfacial stress. Proteins in aqueous solution are known to adsorb to various surfaces. For example, G-CSF (332), a hydrophobic cytokine (174), and IL-2 (333) have been shown to adsorb to glass. The binding of IgG1 to plastic has been reported (334), and BSA, like other proteins, exhibits some propensity to bind to stainless steel (335). Consequently, many biophysical studies have been published on protein adsorption, especially as it applies to processing and protein instability (336–340).

Surface-induced protein instability begins with the adsorption of either native or partially unfolded protein on the surface; this interaction is usually more energetically favorable when a protein is partially unfolded due to their greater exposure of hydrophobic amino acid side chains, which are normally buried within the core of the protein. After initial adsorption of the protein, surface tension forces at various interfaces (i.e., air–liquid interface, solid–liquid interface) can drive aggregation by affecting structural integrity of protein molecules that populate the interfacial region (142,337,341,342). Structural perturbation at the surface combining with desorption of partially unfolded proteins from the surface can lead to nucleation and growth of aggregates in the bulk solution (142,343–346). Thus, interfacial stability of a protein is believed to be dependent on a number of key factors—surface tension, available surface area for adsorption, surface property of a protein molecule (i.e., hydrophobicity), and structural stability (342).

There are an increasing number of reports of proteins undergoing some type of degradation at solid interfaces. This is especially true for membrane interactions, where proteins

undergo aggregation and fouling of membranes (347,348). It appears that the same approaches that stabilize protein against aggregation in solution will work for membrane-induced damage. This includes increasing conformational stability, reducing attractive protein–protein interactions, and using surfactants to limit adsorption of the protein to the surface.

In addition, there are a number of reports of foreign materials causing physical instability in proteins. For example, the shedding of glass nanoparticles during vial depyrogenation led to aggregation in PAFase (349). During filling, metallic particles can be introduced, forming nuclei for aggregate formation (304). Stainless steel nanoparticles can cause aggregation as well (350). A variety of leached materials from rubber, glass and metal components can cause instability in prefilled syringes (351–354), including issues with silicone oil. Silicone oil was implicated in insulin instability in the 1980s (355–357), but was rarely appreciated for causing instability problems for proteins. An extensive study on the effect of silicone oil on protein aggregation found that high concentrations were needed to have an effect (358). Yet, the problem persists. The data suggest that silicone oil may have little effect on a relatively stable protein, but may accelerate aggregation for a protein that is already compromised or marginally stable. This has been seen for an IgG1 where agitation accelerated aggregation in the presence of silicone oil, but silicone oil without the agitation stress did not cause aggregation (359).

Air–Water Interface

Of all interfacial damage, this is the one that appears to be most problematic. For one thing, it is a ubiquitous interface for any product during production. If the final formulation is an aqueous liquid, the opportunity for interfacial damage can occur during storage as well as shipping and handling. Thus, agitation studies are an important facet of screening excipients for a liquid formulation.

There have been a number of agitation studies performed on protein of pharmaceutical interest. The intent is to expose the protein to a sufficient interface to allow damage to occur, if it will. The usual formulation strategy to mitigate damage at the air–water interface is to add surfactants (see below). Agitation is usually accomplishing by stirring or shaking, although vortexing is sometimes reported. For example, extensive aggregation of porcine growth hormone (pGH) was observed upon vortexing for 1 min (360). Addition of 0.1% polysorbate 80 prevented vortexing-induced damage almost completely. Recently, the effect of shaking and stirring on an IgG1 was examined (361). Interestingly, stirring caused much more aggregation than shaking. In both cases, addition of polysorbate 20 was found to be quite effective at reducing interfacial damage. Agitation also caused damage of CNTF (362). In this case, increased stability was observed with the addition of PEG 3350 and propylene glycol as well as polysorbate 20. Therefore, surfactants are quite effective at diminishing interfacial damage of proteins in most systems. While this was well established 20 years ago, we now have many more examples and a greater mechanistic understanding of the stabilization afforded by surfactants. Further details about stabilization of proteins by surfactants are listed below.

Overall, surfactants are the most effective stabilizers with respect to minimizing interfacial damage. The effect of other classes of additives on damage at the air–water interface is less clear. For example, it is now known that increasing conformational stability can decrease the damage that occurs at an interface (363,364). On the other hand, addition of an excluded solute, like sucrose, increases the surface tension at the air–water interface, which can increase the likelihood of conformational rearrangement. Moreover, sucrose could enhance protein adsorption to the air–liquid interface (365) and promote unfolding at the interface (366,367). Therefore, it is not a simple matter to predict the effect of adding an excluded solute, like sucrose, on the interfacial stability of a protein in aqueous solution.

Agitation and Salt Effects

The effects of ions at interfaces have been studied, and such work has implications both for physical instability as well as for how ions affect the protein-solvent interface (368). Less is known about how ions affect protein behavior at the air–water interface. However, recent studies have begun to examine these effects. Enzyme deactivation in aqueous solution and at the gas–water interface in a bubble cell was found to depend on both bubble surface area size and salt concentration, with higher molarity of ammonium formate leading to increased deactivation (369). Salt-specific effects were investigated, and the enzyme was deactivated by chaotropes but not kosmotropes, providing some guidance regarding possible quantitative mechanisms of Hofmeister effects. In addition, formation of insoluble MAb aggregates due to agitation stress was examined in the presence of salts (370). Turbidity was found to increase with the chaotropicity of the added anion and ionic strength. So, there are an increasing number of examples demonstrating that salts can have both positive as well as deleterious effects on interfacial damage. Yet, our mechanistic understanding is still incomplete on this topic.

Ice–Water Interface

The first reports of damage at the ice–water interface appeared in the 1990s (142,371,372). In many cases, the addition of nonionic surfactants reduces the damage significantly (142,373). The extent of protein damage correlates with the surface area of the ice (374). Since then, there have been a number of reports of interfacial damage in frozen systems, including these listed above. One note is that the temperature dependence of the critical micelle concentration (cmc) is often overlooked. The cmc can increase by as much as five-fold upon cooling to near the freezing point of water (374), meaning that what might be a sufficient amount of surfactant for stabilization at room temperature may be insufficient at lower temperatures.

The detrimental effect of multiple freeze–thaw cycles is now well established (375,376). Consequently, nearly all freeze–thaw (F–T) studies are now conducted using multiple (three to ten) cycles. It should also be noted that it is important to use the same cooling and warming methods, as variations in each of these can affect the physical stability of the protein (e.g., references 371,377,378). Even proteins that are usually considered to be highly surface-active, such as IgGs, can undergo aggregation upon repeated F–T cycling (377,378).

Reducing Interfacial Damage of Proteins

In the pharmaceutical industry, nonionic surfactants, especially polysorbate 20 and 80 (also known as Tween 20® and Tween 80®, respectively) are frequently added to protein solutions to prevent or reduce unwanted adsorption and aggregation during storage, filtration, purification, and transportation (142,343,379,380). The ability of surfactants, especially nonionic ones, to reduce interfacial damage is now well established (342). However, it should be noted that the use of nonionic surfactants might be associated with its own set of undesirable consequences. For example, although polysorbate 80 inhibited shaking-induced aggregation of IL-2 mutein, it increased both oxidation and aggregation of the protein during long-term storage (249). Additionally, depending on the temperature and nature of surfactant–protein interaction, nonionic surfactants may foster formation of protein aggregates in bulk solution in a concentration-dependent manner (381).

IMPROVING PROTEIN STABILITY

Our knowledge base on how to improve the stability of a protein is so much larger than in 1989. A few strategies and advances are examined here in some detail. Each topic could be the subject of its own review. However, they are worth noting in the context of protein instability, stabilization and formulation. They include

- ➢ Conformational Stabilization in Aqueous Solution
- ➢ Colloidal Instability
- ➢ Interfacial Instability
- ➢ Drying of Proteins
- ➢ Chemical Modification
- ➢ Site-Directed Mutagenesis

Conformational Stabilization in Aqueous Solution by Excluded Solutes (Osmolytes)

The ability of low-molecular-weight additives to increase the free energy of unfolding was well known in 1989, based on many publications by Timasheff and coworkers (313–315,382,383). However, the application of this approach as a general formulation strategy was only beginning to emerge at that time. Since then, numerous examples of conformational stabilization by excluded solutes have been reported. We now know that osmolytes operate by this general mechanism to provide increased conformational stability to proteins. Thus, nearly any sugar or polyol should increase the structural stability of a protein. In addition, it has also been shown that most amino acids act as excluded solutes (314,383–385), as do a number of salts (384,386) and many polymers, including gelatin (387) and even poloxamers (329).

Beyond increasing the conformational stability of proteins, another value of excluded solutes is to salt proteins out from solution. Such an approach has been widely used for enzymes, which are then sold as ammonium sulfate precipitates. The proteins that are salted out still retain native structure and activity (329,330). Recently, highly concentrated precipitates of native antibodies were prepared by this method (327).

Stabilization by Ligand Binding to the Native State

In contrast to the stabilization afforded by excluded solutes, it is possible to identify ligands that can bind selectively to the native state of a protein, resulting in net stabilization through the Wyman linkage function (388), a concept then elaborated by Tanford (389). Although this idea was known in 1989, it was not widely appreciated by pharmaceutical scientists at the time. Therefore, it is worth highlighting some of the reports that have been made in the last two decades, which demonstrate that many classes of excipients, including surfactants, buffers, polymers, and metal ions, all can improve conformational stability by this mechanism. At the same time, preferential binding to the denatured state can destabilize the conformation of a protein, as shown by Miyawaki (390,391). Consequently, while the Wyman linkage function can explain both conformational stabilization and destabilization, it affords another approach to increase structural stability of a protein. In turn, this should lead to reduced unfolding and subsequent aggregation during storage.

Buffers

The use of buffers to stabilize proteins has often been thought to be solely due to their ability to modulate changes in pH. However, for many protein formulations, especially when the protein concentration is relatively high, the protein, rather than buffer, provides the majority of the buffering capacity (392). In addition, other mechanisms for stabilization from buffers have now been reported (393). They can act as radical scavengers, a fact that goes back to original observations by Good *et al.* (394). These buffers are frequently referred to as Good's buffers. Even more important is the fact that some buffers appear to be able to bind directly to proteins, thereby increasing conformational stability.

Phosphate has been reported to confer some increased stabilization. One possible explanation for this, and many of the observed increases in stability with certain buffers, is direct binding of the buffer to the native state. According to Wyman, this should lead to net stabilization of the protein, provided there is no comparable binding to the unfolded state (388). In the case of phosphate, being highly charged, it is likely that the binding site will be at the N-terminus of an α-helix, where the helix dipole would favor interaction with a negatively charged ligand. Stabilization by phosphate has been reported (395,396).

More often, the stabilization seems to occur with nitrogen-based buffers. The preferential stabilization of an antibody by MES (397) over buffers like citrate has been reported. Similarly, histidine (His) has been shown to stabilize a monoclonal antibody (398), interferon-tau (399), and EPO (400). In the latter case, Tris buffer was also effective. In addition, some stabilization by citrate has been observed. In the case of interferon-alpha (IFN-α), citrate provides greater stability than phosphate or a phosphate–citrate mixture (401). Citrate also stabilizes antitrypsin (402) and IL-1ra (403) as well.

Surfactant

While surfactants mainly stabilize proteins by preventing access to and damage at interfaces (342), it has now been shown that polysorbates can bind to certain proteins, such as human growth hormone (311,376,404). Since those first

reports, polysorbates have been found to bind to fusion proteins as well (405). It is also worth noting that there have been reports of polysorbates not binding to certain proteins, especially antibodies (406,407). Pluronic F-107, another non-ionic surfactant, has been found to bind to G-CSF (408). Ionic surfactants also bind to proteins, conferring stabilization. Binding of anionic surfactants to proteins has been reported for a number of systems, including insulin (409), chymotrypsin (410), BSA (411), and TMV coat protein (412). In all of these cases, direct interaction with the native state of the protein leads to conformational stabilization.

Polymers

There have been a number of reports of anionic polymers (including biopolymers such as heparin) binding to basic proteins, resulting in stabilization. This has been seen repeatedly for aFGF and bFGF proteins (413,414). In these proteins, there is a highly positively charged cleft where the anionic polymer can bind. Other examples of anionic polymers (polyanions) binding to increase protein stability have been reported for insulin (415) and cytochrome c (416).

Cyclodextrins

Cyclodextrins (CDs) are annulated, bowl-shaped sugars that are known to bind small molecules, and a number are now found in approved pharmaceutical products (417). The binding occurs within the relatively hydrophobic interior of the CD, allowing the CD to present a more hydrophilic surface on the outside. In doing so, hydrophobic compounds can be solubilized (418). In the case of proteins, binding to the native state should result in net conformational stabilization, resulting in improved physical stability.

Addition of β-CDs has been reported to increase the physical stability of hGH with respect to aggregation (419,420). In both cases, the CD was shown to bind to the native state of the protein with a millimolar binding constant. In the latter study, it was also shown that the CDs did not improve chemical stability (420). Similar results of improved stability in the presence of CDs have been reported for insulin (421). On the other hand, there has also been a report that CDs decrease protein stability (422). In addition, there is some evidence that CDs may not stabilize simply by increasing conformational stability but play some role as a surface-active agent. It is known that hydroxypropyl-β-CD protects porcine GH from agitation-induced damage (356).

Metal Ions

Many proteins contain metal binding sites. Even a small cluster of acidic amino acids can allow metal binding. Keep in mind that even millimolar binding constants can produce an increase of 1 kcal/mole or more in the free energy of unfolding. For example, it has been known for some time that the four-helix bundle protein, human growth hormone (hGH) can bind a variety of divalent cations (423,424). On the other hand, too much zinc can lead to aggregation of hGH (425).

Similarly, calcium has been shown to provide significant stabilization for the marketed enzyme, DNase (426). The enzyme, fibrolase, contains one mole of zinc. It is not necessary for catalysis, but the zinc atom is critical for the conformational stability of the protein (427). Metal ions, such as calcium, are essential for bridging the two polypeptides chains of Factor VIII (428).

Anion Binding

Stabilization of proteins by direct binding of anions has been observed as well. For example, sulfate appears to bind to endostatin (429) and ribonuclease (430). Ribonuclease has also been shown to bind two moles of chloride ion, resulting in significant structural stabilization (~2–3 kJ/mol) (431). Stabilization of HSA has been observed from binding chloride (432) and carboxylates, such as formate and acetate (433).

Stabilization of Proteins by Ions

The effects of adding salts to a protein solution are many. Some of the effects of are due to specific interactions, such as the binding of cations or anions directly to the protein, as described above. Some of the effects are due to altering colloidal stability through charge screening. At the same time, there has continued to be investigation of the basis for the Hofmeister effect, which first described how the solubility and stability of proteins could be affected by ionic species.

Hofmeister Effects

There has continued to be investigation of the basis for the Hofmeister effect, which first described how the solubility and stability of proteins could be affected by ionic species (434). All in all, our understanding of this behavior has increased since 1989. Therefore, it is worth summarizing what is known about this important aspect of protein stabilization.

Several models have been devised to explain Hofmeister effects on proteins, including those based on the hydrophobic effect (435) and protein-solvent surface tension increments (436). Recently, Broering and Bommarius have challenged the validity of using surface tension increments to predict Hofmeister effects on proteins (437). Instead, the authors suggest that the *B*-viscosity coefficient from the Jones-Dole equation serves as a more accurate predictor of Hofmeister effects. The *B*-viscosity term describes the contribution of ion-solvent effects to solution viscosity (438). For three model enzymes, the authors demonstrate a strong correlation of kinetic deactivation constants with *B* in the presence of anionic chaotropes, whereas a similar correlation using surface tension increments was not found. However, a second study found that a similar correlation did not exist when using chaotropic cations, supporting the claim that anions dominate salt effects in solution (439,440). The most recent work from this group has focused on development of a mathematical model for *B*-dependent Hofmeister effects (441). In another interesting publication, Sedlak *et al.* report a correlation between change in thermal unfolding midpoint with ion concentration and water surface/bulk ion partition coefficients (442). Two proteins with very different net charges at pH 7.0 (−19 and +17) were examined, and both showed similar behavior under the authors' experimental conditions. This suggests that Hofmeister effects are not based on ion–protein electrostatic interaction.

While various predictive models have been described in the literature, recent studies on the fundamental mechanisms behind Hofmeister effects question commonly held views on the behavior of these ions in solution (443). Bulk properties of aqueous solutions, such as boiling point and surface tension, are affected by the presence of ions. Likewise, ionic kosmotropes ("water structure makers") and chaotropes ("water structure breakers") have traditionally been viewed as exerting their effects on proteins through changes in bulk solution properties. To this end, much attention has been directed towards ion solvation by water. Hribar *et al*. modeled ion solvation in a two-dimensional plane using statistical mechanics and Monte Carlo simulations (444). The model was in agreement with Hofmeister series effects and Jones-Dole *B*-viscosity terms. Collins has argued that ionic charge density determines the degree and strength of hydration of an ion and that ion solvation in bulk solution indirectly influences protein solvation (445,446). Theories about long-range water-ordering properties of ions were challenged when Omta *et al*. used femtosecond pump-probe spectroscopy to show that dissolved ions do not affect water structure beyond the first hydrated shell (447). Structuring of water by ions was further investigated by Batchelor *et al*., who used "pressure perturbation" calorimetry to demonstrate a lack of correlation between protein stability and ion hydration (448). While the body of literature devoted to understanding the Hofmeister effect has expanded greatly in the last two decades, a consensus on the mechanisms of this process has yet to emerge.

Colloidal Stability

Protein solutions can be characterized as a population or ensemble of colloidal particles suspended in an aqueous environment. Thus, colloidal stability is a reflection of the energetics of protein–protein interaction that can be manifested in solution properties, such as solubility, viscosity, crystallization and aggregation (449–451). Importantly, the nature of interaction between protein molecules in solution (i.e., attractive *versus* repulsive) can influence the rate of aggregation and the size of aggregates formed (309,452,453). In these studies, osmotic second virial coefficient (B_{22}), a thermodynamic parameter derived from McMillan-Mayer theory, was utilized to provide a quantitative measure of colloidal stability (454). There is an increasing amount of evidence which suggests that, in situations where the structural stability is comparable, reducing the attractive forces between protein molecules (e.g., through charge–charge repulsion) leads to greater physical stability with respect to aggregation (309,450,453,455) For example, Chi *et al*. demonstrated that independent of conformational stability, rhGCSF, a four-helix bundle protein, was maximally stabilized against aggregation when it is in solution conditions that have optimized colloidal stability (455).

Stabilization by Drying

One means of improving protein stability is lyophilization, also called freeze-drying, although other drying methods for proteins have been reported (see below). Despite the increased cost of production of lyophilization, this process can often provide advantages for shipping, long-term storage stability, and improved stability to temperature excursions. However, as with any protein formulation, there must be a good rationale for the additives that are selected. Since 1989, our understanding about developing lyophilized formulations has increased tremendously, so that, by 1997, the rules for rational design of stable lyophilized protein formulations were published (456) and then later expanded (457).

Freeze-drying (lyophilization) can produce instability in proteins at each step of the process. These include cooling, freezing, primary drying, and secondary drying. Details of each of these stages of freeze-drying can be found elsewhere (458–460). In short, lyophilization can be considered a combination of two distinct stresses: freezing and drying.

During freezing, the protein can be damaged by a variety of mechanisms, emphasizing the importance of controlling the freezing stage during lyophilization (461). There is the possibility that a pH shift can occur during freezing as a result of selective crystallization of buffer salts. While this was known for sodium phosphate since 1959, a number of newer studies have illustrated further how various buffers tend to crystallize during freezing (462–465), including phosphates, succinate, and tartrate (466). Citrate has also been shown to acidify to as low as pH 3 during freezing (467). This acidification arises due to selective crystallization of one of the buffer components. In general, the effect is minimized or eliminated in the presence of additional solutes or by increasing the protein concentration.

Also, freeze concentration can occur during the freezing process, especially for larger sample volumes. This is particularly problematic for large volumes of bulk drug (468,469). Deleterious effects of freeze concentration can result from the enormous increase in protein concentration and ionic strength in the non-ice phase of the formulation. This freeze concentration will lead to an increase in protein interactions, potentially leading to an increase in protein aggregation. This can often be inhibited by use of a preferentially excluded solute such as sucrose. Sucrose or other disaccharides can improve the stability of the protein during freeze-concentration by means of preferential exclusion (295,315,470).

Third, during freezing, proteins are exposed to a large ice–water interface. This presents a challenge for proteins that are surface labile (371,372). Therefore, many excipients, such as non-ionic surfactants, are added to the formulation to prevent surface-induced degradation. Polysorbate 20 and polysorbate 80 are commonly used for this purpose (142,295,375,462). Fourth, for many formulations cold denaturation could occur, provided the denaturation temperature is near −25°C or above. This instability was discussed above.

During the subsequent stages of lyophilization are the water removal processes of primary and secondary drying. Briefly, during primary drying, the water is removed in the form of ice in a process of sublimation under vacuum. In secondary drying, the bound residual water is removed under vacuum. This requires a higher temperature than for primary drying, which may be why the stress placed upon a protein is greater during secondary drying than during primary drying (373). Nevertheless, during the drying stages of this process, water is removed from the formulation. These drying stresses can often lead to dehydration-induced structural changes. However, these dehydration-induced structural perturbations can be often minimized by use of formulation additives, such

as disaccharides (470–472). Many labile proteins are protected from dehydration-induced degradation by a mechanism referred to as the water replacement mechanism, in which disaccharides are able to hydrogen bond to the protein, thus preserving the secondary structure of the protein (470–472). Hydrogen bonding of additives to polar and charged groups has been shown to protect proteins in cases of lysozyme (470), α-lactalbumin (473), and many other examples (470–472). The maintenance of the secondary structure of the protein has been shown to be a critical parameter for the successful development of a freeze-dried formulation, as it seems to correlate well with storage stability (474–477).

Stability during long-term storage typically requires a lyoprotectant to replace the hydrogen binding lost with removal of water and to provide a glassy matrix that limits mobility. Usually, disaccharides are used, such as sucrose, trehalose, or maltose. Even though these sugars are similar in size, they display very different solid-state properties in terms of molecular mobility and glass transition temperature (T_g) (478,479). Simply having a higher T_g is not sufficient to provide increased storage stability (480), although many studies have found a correlation between T_g and storage stability (474,477,481). Increasing the T_g by adding high-molecular-weight polymeric additives is usually ineffective due to phase separation of the protein and polymer (482).

Numerous studies have examined the differences between sucrose and trehalose in the stabilization of proteins in the solid state (483–485). Many reports on lyophilized myoglobin have shown trehalose to be superior due to greater coupling between protein and matrix relative to sucrose or maltose (483,486,487). This may be due to stronger water-mediated hydrogen bonding between protein and sugar (483) or lack of nanophase separation (484). More likely, it is due to modulation of β-relaxation processes in the solid state. In recent years, Cicerone and coworkers have demonstrated that these higher frequency relaxation processes appear to be critical in controlling long-term stability (488–490). Interestingly, there does not appear to be a correlation between lower frequency modes in the solid state (termed α-relaxation) and storage stability (491,492).

These concepts of solid-state mobility and structural relaxation are connected to other emerging ideas for stabilization of lyophilized proteins. First, the work of Cicerone and coworkers (488), along with observations from other laboratories, has demonstrated that small amounts of low molecular weight compounds, called plasticizers for their ability to lower T_g, can provide improved stability despite compromising T_g. This has been shown for water (115,492), glycerol (488), and sorbitol (492). The degree to which plasticization will improve stability may depend on properties of the protein, such as lack of disulfides and the percentage of polar groups on the surface (493). Second, it appears annealing the frozen matrix prior to primary drying can improve stability as well (494,495). Annealing provides a number of benefits, including reducing intervial heterogeneity and possibly reducing primary drying times (496). Overall, our understanding of the relationship between solid-state properties, protein stability and structure have increased in the last 20 years.

A couple other aspects of lyophilization development should be mentioned. First, it is useful to consider that the formulation and lyophilization cycle are well matched to ensure maximal product quality. This means having a cycle that is not only efficient, but also produces a pharmaceutically elegant cake, as well as a stable product. Elegant cake structure is often achieved by the use of bulking agents, which are additives that crystallize to provide mechanical rigidity to the cake. These include compounds such as mannitol or glycine (142,497,498). Amorphous bulking agents, such as hydroxyethyl starch, have also been reported (499). Recently, it has been found that bulking agents also have an impact on stability (477), presumably by having a small amount remain amorphous and plasticizing the matrix. Second, there are recent reports of material other than sugars yielding glassy matrices that might be used for embedding proteins and achieving stable dosage forms. These include many of the naturally occurring amino acids (500–502). In addition, combinations of compounds provide glassy matrices that have properties superior to the individual components. These include amino acids and polycarboxylic acids (503), LiCl and trehalose (504), and Arg in the presence of organic acids (505). This approach appears to work if there are strong interactions between the two components, such as electrostatic attraction or extensive hydrogen bonding (506).

Other Drying Methods

Several other drying methods have been examined for their ability to stabilize proteins. For bulk powders, there have been a number of reports using spray drying (507–510). These include formation of stable powders of IgGs (511,512) and hGH (513). Another approach is called spray freeze-drying, where droplets of the protein solution are frozen in liquid nitrogen and the subsequent particles are freeze-dried to remove the water (514–518). This approach uses a standard lyophilizer, but also requires equipment for conducting the spray-freezing process.

Both air drying (470) and vacuum drying (500,519) have also been reported for proteins. For example, IFN-α has been vacuum dried and reconstituted with nearly complete recovery of structure and activity (520). On the other hand, film drying (air drying of a protein solution, forming a film) has been reported for hGH (521). Also, supercritical fluid drying has been widely reported (522–525). A comparison of the various drying methods that have been used for protein formulation has been published (526).

Site-Directed Mutagenesis

Current recombinant DNA technology enables scientists to make specific and rational changes to the primary sequence by means of site-directed mutagenesis (527,528). Certainly, mutagenesis can be used to improve the solubility of the protein. For example, using portions of the hGH sequence, the solubility properties of bovine growth hormone (bGH) were improved by site-directed mutagenesis (529,530). Another example of site-directed mutagenesis to enhance the physical stability of a protein is the in the case of leptin. In these studies, Ricci and coworkers examined several mutations aimed at minimizing aggregation and precipitation occurring near neutral pH (531). One of the driving forces for the aggregation of leptin is believed to be one or both of

the surface-exposed tryptophans. By making numerous mutations to enhance the physical stability of leptin, making mutants with increased neutral pH solubility was achieved. It has also been shown that improving the stability of beta-turns through mutagenesis increased the conformational stability of proteins (532).

Mutagenesis has been used to improve the chemical stability of proteins as well. The IgG-binding domain of streptococcal protein G was modified by mutagenesis. Here, the investigators were able to enhance the protein's stability in alkaline conditions by replacing amino acids susceptible to high pH, asparagines and glutamine, with amino acids that are less susceptible to degradation under basic conditions (79). As with the mutation of a labile residue such as Asn for a protein under alkaline conditions, similar mutations could be considered for the enhancement of stability of a protein susceptible to oxidation. Lu *et al.* elegantly demonstrated the use of site-directed mutagenesis of methionine residues of GCSF to investigate the impact of oxidation on the stability and activity of G-CSF (204). In addition, Kim *et al.* reported an example where mutations were made to staphylococcal nuclease in order to decrease its lability to oxidation (207). However, these investigators not only studied the effects of methionine mutations on protein oxidation, but also to the conformational stability determined by guanidine unfolding between oxidized, non-oxidized, and the various mutations.

Finally, mutagenesis has been widely employed to improve the physical stability of proteins, especially with respect to aggregation. A few examples will suffice to demonstrate this point. Numerous studies on the use of mutagenesis to stabilize antibodies have appeared. Using an aggregation propensity mapping algorithm, a number of more stable mutants of full-length antibodies were designed and prepared (533–536).

Computational Methods and Protein Stability

In the past two decades, numerous algorithms have been developed to predict the aggregation behavior of proteins. A recent book chapter summarizes these methods (537) and the current advances in this area. In general, there are numerous algorithms available to predict aggregation propensity from the primary sequence. There are also methods to make predictions based on the overall properties of the protein (e.g., hydrophobicity, pI, *etc.*) (e.g., references 538 and 539).

In addition, researchers are continuing to advance our knowledge of protein stability by combining tools from computational chemistry and structural biology to design proteins that have specific characteristics, such as decreased immunogenicity (540,541), improved activity (542), and enhanced stability (534,543). Additionally, computational methods have the potential to play a large role in protein stabilization, such as optimizing the electrostatic field on the surface (544). In addition, Dahiyat has eloquently described other such examples of *in silico* design of protein surfaces and mutations occurring in the boundary between the core and surface (545). Space limitations do not permit a full description of all of the activities in this field. The use of computational methods is certain to improve the design of stabilization studies as well as provide greater mechanistic insight into the stability of proteins.

Chemical Modification

In many cases, the specific protein may not be amenable to changes in the primary sequence due to decreased activity or other significant manifestations of the behavior of the protein. Moreover, post-translational modifications, many of which occur *in vivo*, can alter the properties of proteins in profound ways (546). Therefore, controlling the extent of modification, such as with glycosylation, can provide a means of modulating the chemical and physical stability of a protein. These modifications can be accomplished *in vivo* (by controlling fermentation and using molecular biology methods) or *in vitro*.

Among synthetic methods, the most common method to modify proteins is by the addition of polyethylene glycol (PEG) groups (547) in a process referred to as pegylation. While the initial intent was to extend the half-life of a protein *in vivo*, it is now known that pegylation can improve the conformational and physical stability of a protein. For example, interferon-α_{1b} was stabilized by the addition of PEG groups in a site-specific manner (548). Similarly, pegylation has been found to stabilize trypsin (549), chymotrypsin (550), endostatin (551), and single chain antibody fragments (552).

There is certainly a strong rationale for employing glycosylation of proteins based upon lessons learned from nature. Glycosylated proteins are certainly very prevalent in biological systems. It has been reported that close to half of all proteins are glycosylated (553). The role of glycosylation in the stabilization of proteins was recently reviewed by Solá and Griebenow (554). Their review contains a detailed summary of how protein glycosylation affects the physical stability of proteins, many of pharmaceutical relevance. In the case of protein solubility, it has been proposed that the addition of glycans to the protein via chemical glycosylation can enhance the solubility by increasing the degree of glycosylation and by increasing the surface-accessible surface area (555,556). Glycosylation can also improve chemical stability. In the case of erythropoietin (557), the glycosylated form displayed an enhanced stability with respect to tryptophan degradation compared to non-glycosylated erythropoietin. Even the attachment of a sugar via glycation can improve the physical stability of a protein. It has been shown that the glycation of proteases (trypsin, chymotrypsin) improves its thermostability (558).

A variety of approaches for stabilization of enzymes have been summarized by Fagain (559). This included a number of cross-linking studies and even chemical modification of Lys residues (560), which presumably increased solvation and colloidal stability. The crosslinking could involve introduction of a disulfide bond or could be accomplished by chemical reaction of surface acidic and basic groups using linkers.

INTERRELATIONSHIP BETWEEN CHEMICAL AND PHYSICAL INSTABILITY

Although it is convenient to distinguish chemical from physical instabilities for purposes of discussion and mechanism, the fact remains that chemical and physical instabilities are interrelated. Our understanding of this has continued to improve. This section describes how certain chemical degradation processes make a protein more prone to aggregation. Likewise, there are examples of denaturation increasing the chemical reactivity of a protein.

Connection Between Deamidation and Aggregation

Deamidation has been found to produce species that are more prone to aggregate than the unmodified protein. For example, deamidation of βA3-cystallin leads to destabilization of the native structure and increased aggregation (561,562). Deamidation has also been linked with decreased solubility of lens crystallins (563) and to a lower kinetic barrier for unfolding (100). The collagen-to-gelatin transition appears to be affected by deamidation (564).

Aggregation of βB1-crystallin is accelerated by deamidation at both Asn and Gln residues (565). The stability in urea, with respect to deamidation, is decreased as well (566,567). Deamidation appears to lead to increased fibril formation in Aβ peptides (568,569). Also, deamidation may lead to increased amyloid propensity in β2-microglobulin (570).

Connection Between Glycation and Physical Stability

Recently, it has been shown that glycation can increase thermostability (558). Whether this is a general phenomenon is not clear. Rearrangement of the initial Schiff base leads to what are referred to as advanced glycation end products (AGEs). These materials have been shown to exhibit increased aggregation propensity in some cases (571).

Correlation Between Oxidation and Physical Stability

It is important to note that oxidation can lead to increased propensity for physical instability, such as aggregation. This has been seen for an IgG1 (210), α-synuclein (572), calcitonin (573), trypsin inhibitor (574) and fumarase (575). Moreover, it has been shown that oxidation can reduce the conformational stability of a protein, as with glutamine synthetase (576), calmodulin (577), and a Fc fragment from an IgG1 (210).

SUMMARY

The field of protein stabilization and formulation has made tremendous progress in the past 20 years, and this review only contains a portion of the relevant studies. Our increased understanding of chemical instability pathways now allows us to adjust solution conditions to minimize degradation. Stabilization by excipients seems to be due to the mechanisms first espoused by Timasheff and Wyman. Now we have a large number of examples of how proteins can be stabilized in aqueous solution using these general approaches. Our understanding of stabilization of proteins in dried solids has evolved as well, with attention now turning to the molecular details of interactions in the solid state. Interfacial damage continues to be a challenge, and we are just beginning to appreciate how various excipients can modulate behavior at interfaces. Finally, it is clear that chemical and physical instability are linked in many systems. Overall, this field has advanced so that rational design of dried and liquid formulations is more possible than ever before. Yet, we continue to find new facets of protein behavior as we apply the tools of computational chemistry, biophysics, and molecular biology. This suggests that the next 20 years will continue to provide improved insight and knowledge regarding the stability of pharmaceutical proteins.

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