STABILITY OF SOLID PHARMACEUTICAL PROTEINS

by

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ABSTRACT

The solid-phase deterioration of three representative pharmaceutical proteins, recombinant human albumin, insulin, and tetanus toxoid vaccine, was investigated herein. These studies were conducted by incubating solid (e.g., freeze-dried) proteins at elevated temperatures (≥37°C) and various moisture levels via sorption from the vapor phase (relative humidities ranging from 11-97%). For all three proteins, storage in such conditions (which mimic those for proteins loaded within a polymeric depot intended for sustained release) resulted in marked aggregation as revealed upon subsequent reconstitution in aqueous media. The chemical pathway for this moisture-induced aggregation depended on the protein and its environment. The mechanism for the aggregation of recombinant human albumin was found to be thiol-disulfide exchange between the lone free thiol of cysteine and the seventeen disulfide bonds present in the native structure. Insulin, which has no free thiols, was found to undergo a disulfide interchange catalyzed by low-molecular-weight thiols formed from the β-elimination of an intact disulfide. In addition, insulin solid-state aggregation occurred via non-covalent interactions. The routes of solid tetanus toxoid aggregation include both these and also another covalent, non-disulfide pathway. This latter is hypothesized to occur via a formaldehyde-linked Shiff base intermediate (formaldehyde was added for the detoxification of the protein), resulting in cross-links primarily among lysine, histidine, and tyrosine residues.

In all three cases, the extent of aggregation was critically linked to the moisture content. At low levels (e.g., near or below the theoretical water monolayer coverage), complete stability was observed. As the amount of bound water was raised, aggregation ensued as a result of increased conformational mobility of the protein molecules. Recombinant human albumin and tetanus toxoid exhibited bell-shaped dependencies of the rate of aggregation on the water content, with the maxima at some 50 g water sorbed per 100 g dry protein. In the case of recombinant human albumin, the dramatic decrease in aggregation at higher moisture levels was hypothesized to be the result of a refolding during rehydration; this protein was found to undergo a partial and reversible unfolding upon dehydration, as revealed by Fourier-transform infrared spectroscopic analysis of secondary structure in the amide III region.

The knowledge that solid protein aggregation depends on the level of moisture was used to devise more stable protein formulations. Among nineteen different compounds tested as stabilizers for recombinant human albumin (when added to the aqueous solution prior to lyophilization), the most potent were the strongest water-sorbers, drawing in water from the humidified air and bringing it in the vicinity of the protein. Other rational stabilization strategies were developed to protect against moisture-triggered aggregation based on the elucidation of the chemical pathways. Recombinant human albumin was stabilized by lowering the pH of the aqueous solution prior to lyophilization to insure protonation of the cysteine residue. This approach also succeeded in the case of insulin, since β-elimination is catalyzed by hydroxide ions. Another highly successful strategy to stabilize insulin was the addition of Cu^{2+} which catalyzed the oxidation of low-molecular-weight free thiols, rendering them incapable of catalyzing disulfide interchange. The stabilization of lyophilized tetanus toxoid in the presence of moisture was accomplished by either succinylating the vaccine to block reactive amino groups or by treatment with cyanoborohydride to reduce the reactive formaldehyde-linked Shiff base species. The success of these approaches demonstrates how mechanistic understanding of protein deterioration can be used to rationally improve the stability of solid protein formulations.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Modern biotechnology has made feasible the large-scale production of proteins and peptides for a variety of therapeutic and diagnostic applications. Annually, biopharmaceutical drugs now have total sales of over $6 billion (Thayer, 1995). The potential is even greater. However, it is being hampered by the fact that the development of a new pharmaceutical protein on average takes about ten years and may cost more than $350 million dollars (DiMasi *et al.*, 1991; Thayer, 1995).

The successful exploitation of such biopharmaceuticals requires preservation of biological activity in all steps of development including storage and delivery. Compared to traditional low-molecular-weight drugs, proteins pose additional challenges in preserving this activity because they are much larger, contain relatively labile groups, and possess fragile three-dimensional structures (Manning *et al.*, 1989; Volkin and Klibanov 1989; Cleland *et al.*, 1993).

To ameliorate this stability problem, it is common practice to formulate proteins as solids (usually lyophilized, or freeze dried) and, in some instances, suspensions within polymer matrices intended for sustained release (Langer, 1993). (This latter delivery mode has been proposed for pharmaceutical proteins exhibiting short half-lives when administered orally and thus requiring frequent bolus injections.) However, even this may not provide the necessary stabilization, since it has been recently found that solid proteins are susceptible to deterioration, particularly aggregation, in the presence of moisture (see Chapter 2). For instance, the controlled delivery of insulin from a polymeric device *in vivo* is hampered by problems with regulation of release rate due to instability of insulin in the polymer matrix when exposed to elevated temperature and moisture (Brown *et al.*, 1986). Thus, it is important to study protein deterioration in the solid state, especially since only limited information is available compared to the wealth of knowledge on their deterioration in water.

During the transition from a dry, solid protein (with bulk water removed) to the fully hydrated one, profound changes occur in protein conformation, dynamics, and other properties that have repercussions for stability in the solid state (Hageman, 1988, 1992; Rupley and Careri, 1991). In general, proteins are more stable in the solid form than in aqueous solution. For example, lyophilized ribonuclease in the dry state or suspended in anhydrous solvents exhibits appreciable stability even at temperatures well above 100°C, whereas in aqueous solution rapid enzyme inactivation ensues (Volkin *et al.*, 1991). The hydration of solid proteins accelerates deleterious processes due to greater conformational flexibility of the protein molecule and higher mobility of reactive species. This effect is important for pharmaceutical applications (Chapter 3).

Herein, the solid-state behavior of three therapeutic proteins was examined. When stored at elevated temperature and humidity, recombinant human albumin (Chapter 4), insulin (Chapter 5), and tetanus toxoid (Chapter 6), all formed intermolecular cross-links, with the pathway depending on the nature of the protein and its environment. Rational stabilization strategies have been designed to target these elucidated pathways, leading to the development of more stable protein formulations.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

The diverse biological activity of peptides and proteins has led to their growing use as pharmaceuticals. However, the formulation and delivery of these biopharmaceuticals are problematic due to their relative fragile nature compared to traditional, low-molecular-weight drugs. For this reason, proteins are often formulated in the solid state to increase their stability; biopharmaceuticals, like other drugs, generally exhibit superior stability in the solid phase. In addition, certain delivery devices, such as those designed for sustained release from polymeric matrices, contain solid protein. Thus, it is important to examine the stability of solid proteins, particularly under pharmaceutically relevant conditions.

In this chapter, mechanisms of protein inactivation will be introduced, with emphasis on those which are most relevant to solid protein systems. In addition, issues will be discussed relevant to the storage, shelf-life and delivery of solid pharmaceutical proteins. Finally, perspectives will be presented on rational approaches for stabilization of solid proteins, in particular against the phenomenon of moisture-induced aggregation.

2.2 Mechanisms of Protein Deterioration Relevant to Solid Systems

Recent, detailed reviews of the various modes of protein deterioration can be found elsewhere (Manning *et al.*, 1989; Volkin and Klibanov, 1989; Cleland *et al.*, 1993; Schwendeman *et al.*, 1995a). The deleterious processes occurring to proteins can be divided into chemical instability pathways and physical instability pathways (Table 2.1). Many of these processes have been reported to occur to solid therapeutic proteins either within polymeric matricies or under conditions directly relevant to their formulation in such a system.

Table 2.1

Some deleterious processes occurring to proteins

Conformational (non-covalent)

- Reversible unfolding
- Irreversible aggregation via non-covalent interactions (precipitation)

Chemical (covalent)

- Deamidation
- Hydrolysis
- Oxidation
- β-Elimination
- Incorrect disulfide formation (thiol-disulfide interchange and thiol catalyzed disulfide interchange)
- Maillard reaction
- Transamidation

2.2.1 Protein conformation, unfolding and non-covalent aggregation. The sequence of amino acids of a polypeptide chain (primary structure) can form various secondary structures such as α-helices and β-sheets. Typically, more than half of the native (folded) protein structure is composed of these elements; the remainder may be in other secondary structures such as loops, turns, and random coil (Creighton, 1983; Privalov, 1992). In turn, these elements fold into the overall three-dimensional organization, known as the tertiary structure.

Each protein has an exquisite tertiary structure which is critical to its function. This structural integrity is compromised upon unfolding, or denaturation:

$$N \leftrightarrow U$$

where N and U represent native (folded) and unfolded (denatured) conformations. In general, the protein surface has a higher density of charged and polar residues, which can interact with water and other polar solvents, whereas hydrophobic residues are tightly packed within the protein core and are excluded from the solvent (Creighton, 1983). Usually, the unfolded species has little or no bioactivity relative to the native conformation and unfolding can also accelerate other (covalent) pathways of deterioration (Manning *et al.*, 1989; Volkin and Klibanov, 1989; Cleland *et al.*, 1993). Some proteins also exhibit an intermediate species upon unfolding:

$$N \leftrightarrow I \leftrightarrow U$$

where the intermediate species, I, contains both folded and unfolded domains and may be involved in other destabilization pathways. However, such intermediate species are difficult to isolate and identify.

Various forces have been elucidated in stabilizing the native state. Briefly, these include electrostatic interactions, hydrogen bonding, van der Waals interactions, and hydrophobic interactions (Dill, 1990; Creighton, 1983; Privalov, 1992). Typically, the overall structure is stabilized only slightly; typically, the Gibbs free energy for the folding equation is only about only 5-50 kcal per mole (Privalov, 1979, 1992; Pace, 1990). Thus, only a moderate change in the protein's environment can lead to the disruption of the delicate balance of forces involved in stabilizing protein conformation.

Protein unfolding usually leads to the exposure of hydrophobic moieties previously buried in the protein interior. This unfavorable situation (of hydrophobic groups in contact with aqueous solvent) can be ameliorated by the association of unfolded molecules via non-covalent interactions to form aggregates. Ultimately, these aggregates can reach such a large molecular weight that they are no longer soluble, which is observed macroscopically as precipitation. Even though unfolding is a reversible process, aggregation is practically irreversible and usually leads to a loss of soluble, folded monomeric protein. In addition to binding to each other, unfolded proteins may bind to hydrophobic surfaces such as that present by a hydrophobic polymer and this event may also promote non-covalent aggregation (Thurow *et al.*, 1984; Sluzky *et al.*, 1992).

In a study of several proteins in the solid (lyophilized) form, Liu *et al.* (1991) found that some proteins (for example, β-lactalbumin) were prone to aggregation by non-covalent forces in addition to other covalent pathways while others (e.g., ovalbumin) aggregated solely by the former route. This study was conducted by incubating a wetted, lyophilized protein at 37°C.

2.2.2 Dehydration-induced conformational changes. The structure of protein following the lyophilization process has been a topic of debate, even though theoretical considerations suggest that dehydration should result in significant conformational changes (Kuntz and Kauzman, 1974). For example, Rupley and co-workers (Rupley *et al.*, 1983; Rupley and Careri, 1991) have reviewed data regarding lyophilized lysozyme obtained by various solid-state techniques (including IR spectroscopy and hydrogen isotope exchange) and concluded that the conformation of the solid protein is the same as in aqueous solution. In contrast, dehydration-induced conformational changes have been observed for several other proteins by IR spectroscopy (Prestrelski *et al.*, 1993a, 1993b). Furthermore, a recent report on bovine pancreatic trypsin inhibitor has discovered that this protein undergoes a partial (and reversible) denaturation upon freeze-drying, as determined by hydrogen isotope exchange/high-resolution NMR (Desai *et al.*, 1994). Indeed, several recent investigations have confirmed the view that removal of water from proteins leads to significant (reversible) structural rearrangement (Griebenow and

Klibanov, 1995; Dong *et al.*, 1995; Costantino *et al.*, 1995a, 1995b). For example, Griebenow and Klibanov (1995) have uncovered via FTIR that for some dozen proteins, the removal of water results in a significant decrease in α-helical structure with a rise in β-sheet structure. The β-sheet structure may be favored because of the low degree of solvation it requires (Barlow and Poole, 1987).

2.2.3 Deamidation. In addition to the unfolding event and the ensuing related events of adsorption and aggregation/precipitation, proteins also may exhibit a variety of chemical (covalent) processes leading to their loss of activity. One such instance is the deamidation of asparagine (Asn) and glutamine (Gln) residues, which have been observed for numerous therapeutic proteins under a variety of conditions (Clark *et al.*, 1992). Asn residues, particularly those adjacent to glycine (Gly) residues, are most susceptible. This process occurs most often at neutral to alkaline conditions. Under these conditions, the proposed mechanism of deamidation is via an intramolecular rearrangement to form a five-membered succinimide ring, with the liberation of ammonia, as depicted in Figure 2.1. This succinimide ring is rather unstable, and undergoes hydrolysis and racemization. Thus, the possible products are L-,D-aspartyl and L-,D-isoaspartyl (β-aspartyl) peptides. In addition to deamidation occurring via the succinimide intermediate, deamidation may occur at acidic conditions (pH 1-2) by direct hydrolysis of the side chain amide; however, this process occurs at a much slower rate than the succinimate-intermediate pathway at neutral to alkaline conditions.

Regardless of which product (either aspartyl or isoaspartyl) is generated, deamidation has the consequence of introducing a new positive charge (i.e., free carboxylic acid) to a protein. This may or may not affect the bioactivity, depending on the location on the protein. For example, it has been determined that the deamidation of peptide growth hormone releasing factor leading to aspartyl and isoaspartyl forms reduces the bioactivity by 25- and 500-fold, respectively, compared to native (asparaginyl-containing) peptide (Friedman *et al.*, 1991). In contrast, deamidation occurring at residue Asn^{149} in another therapeutic protein, recombinant human growth hormone, did not significantly affect the biological activity (Becker *et al.*, 1988).

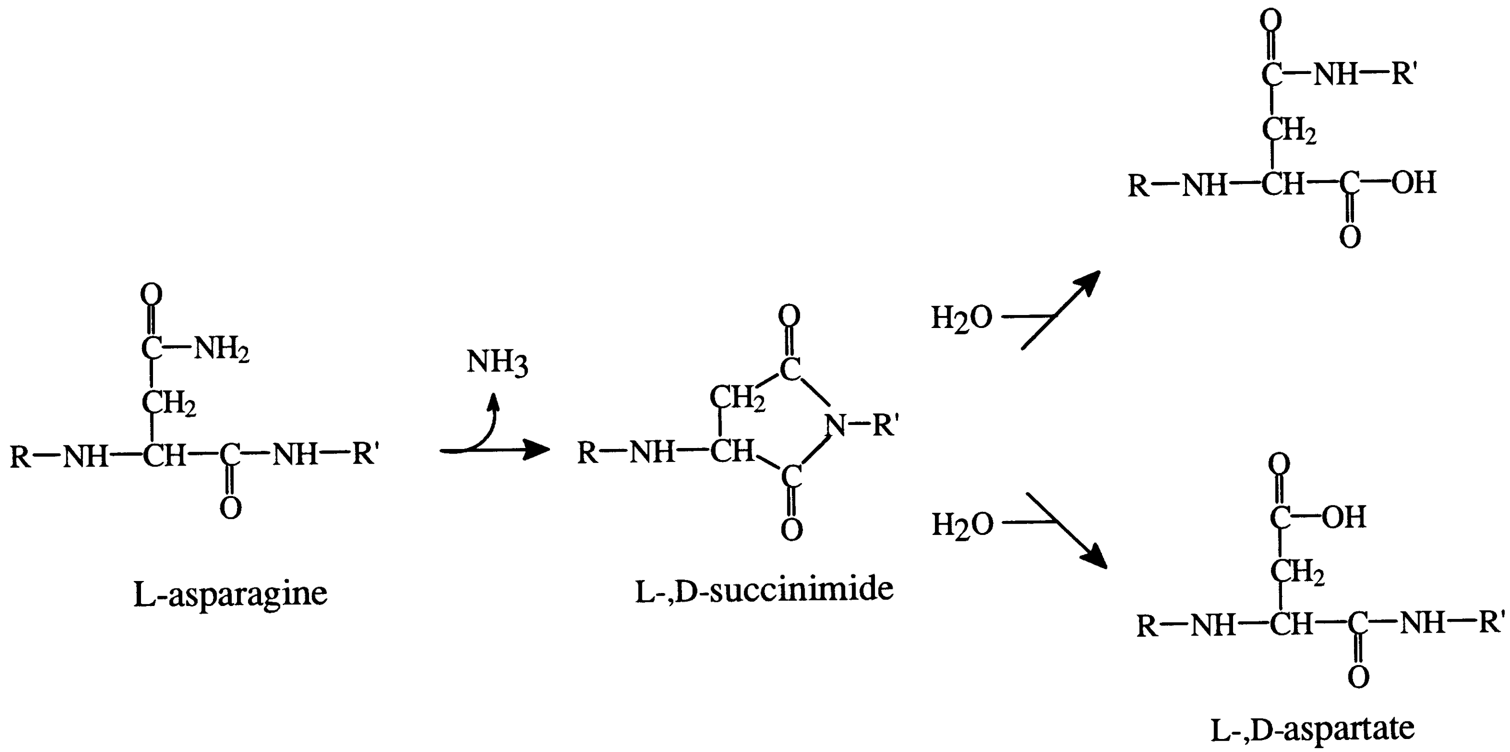


Figure 2.1 Chemical pathway for deamidation of asparagine residues. A similar pathway may be envisioned for glutamine residues, except that the reaction proceeds via a six-membered succinimide ring, rather than the five-membered one shown.

Deamidation has been observed occurring to solid protein and peptides upon exposure to moisture and elevated temperatures, conditions which are relevant to their delivery from polymeric systems (Costantino *et al.*, 1994b; Schwendeman *et al.*, 1995b). For example, Hageman and co-workers (1992a) have found some deamidation occurring to lyophilized recombinant growth hormone incubated at 30°C and 96% relative humidity. In another study, it was found that for a lyophilized model peptide, deamidation occurred under various solid-state conditions (Oliyai *et al.*, 1994). These studies indicate that deamidation may be a problem for solid proteins or peptides suspended within polymeric matricies intended for sustained-release *in vivo*.

2.2.4 Hydrolysis. Under acidic conditions, the peptide backbone may hydrolyze. Near aspartate (Asp) residues, this hydrolysis is about 100 times faster than when adjacent to other peptide bonds (Schulz, 1967). When Asp is adjacent to proline (Pro), the hydrolysis is particularly prevalent (Piszkiewicz *et al.*, 1970). Cleavage can occur at either the C-terminal (via a five-membered ring intermediate) or the N-terminal (via a six-membered ring intermediate) peptide bond of Asp. The mechanism for the former case is shown in Figure 2.2. Note that the carboxyl group of Asp acts as an intramolecular catalyst. A similar mechanism is proposed for N-terminal peptide bond hydrolysis.

Considering the work done with enzymes such as ribonuclease A and lysozyme (Ahern and Klibanov, 1985; Zale and Klibanov 1986), it is apparent that the significant loss of protein structure accompanied by hydrolysis will result in significant loss of a therapeutic protein's biological activity. Thus, hydrolysis may be a concern for solid proteins and peptides loaded within polymeric systems, especially if such systems are composed of bioerodible acidic monomeric units (Schwendeman *et al.*, 1995a). In this case, the microenvironment experienced by the protein within the matrix during release would facilitate hydrolysis. A further note is that therapeutic proteins, such as recombinant bovine growth hormone (Hageman *et al.*, 1992a), as well as model peptides (Oliyai *et al.*, 1994) have been shown to undergo hydrolysis in the solid state.

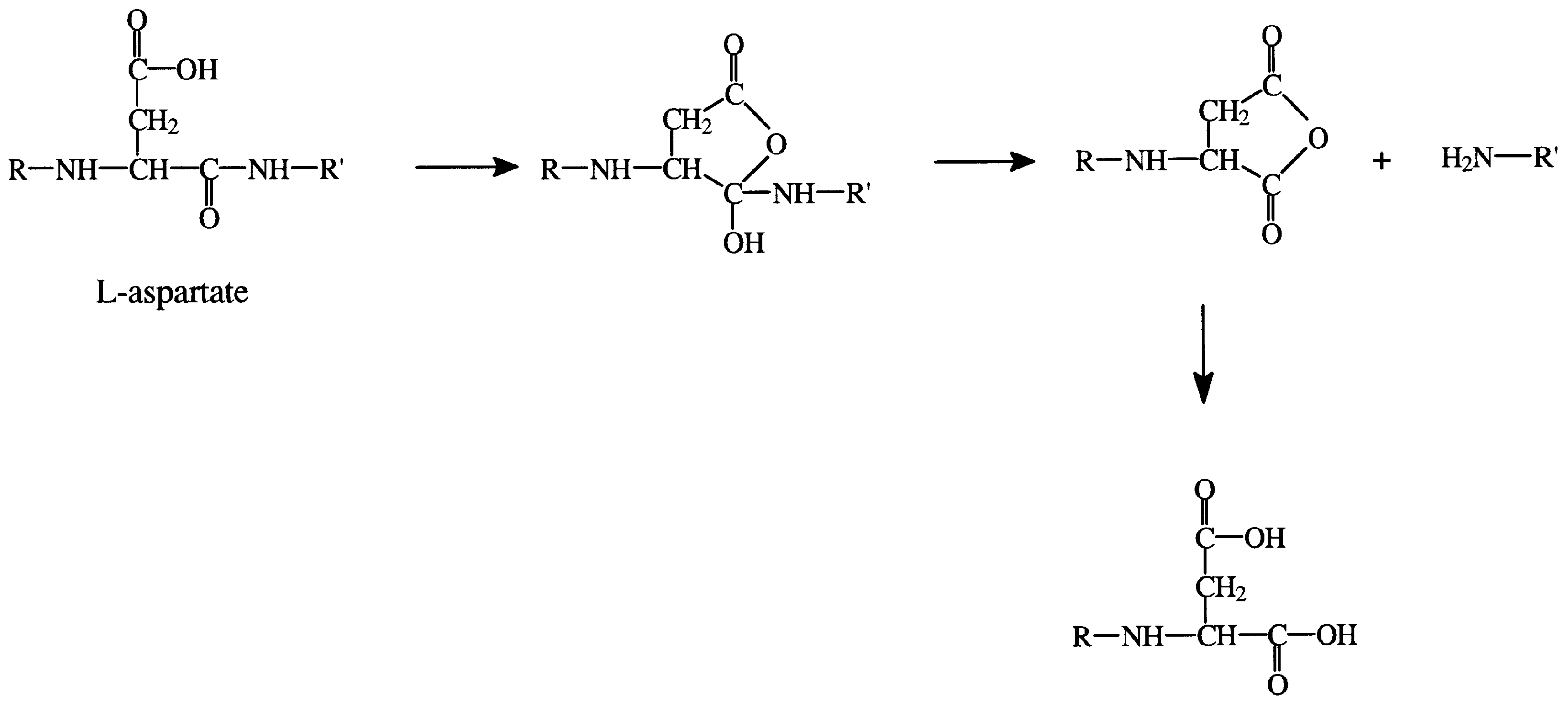


Figure 2.2 Hydrolysis of the peptide backbone. Shown is hydrolysis of the C-terminal peptide bond adjacent to an aspartic acid residue. Similarly, N-terminal peptide bond hydrolysis occurs via a six-membered ring intermediate.

2.2.5 Oxidation. Various residues such as methionine (Met), cysteine (Cys), histidine (His), tryptophan (Trp) and tyrosine (Tyr) are all conceivably susceptible to oxidation, particularly in the presence of oxidizing agents such as hydrogen peroxide. Of these, Met and Cys are probably the most likely to experience oxidation under conditions relevant to solid protein loaded within polymeric systems. At mildly acidic conditions in the presence of hydrogen peroxide, Met is the primary residue experiencing oxidation to form Met sulfoxide (Figure 2.3). Like deamidation of Asn or Gln, the oxidation of Met may or may not affect the biological activity of a therapeutic protein. For example, it has been reported that oxidation of Met^{14} and Met^{125} in human growth hormone does not significantly alter its biological potency (Teh *et al.*, 1987; Becker *et al.*, 1988). In contrast to the effect upon human growth hormone, for another, related protein, chorionic somatomammotropin, oxidation occurring at Met^{64} and Met^{179} resulted in drastically decreased biological potency (Teh *et al.*, 1987). Note that for human growth hormone the oxidation of Met^{125} to the sulfoxide derivative has been observed in the lyophilized powder exposed to light (Pearlman and Nguyen, 1992).

Residues of Cys are also prone to oxidation, yielding various forms such as disulfide, sulfenic, sulfinic, and sulfonic acids, depending upon the conditions (Manning *et al.*, 1989). Because oxidation rates are greater for the thiolate ion (deprotonated) compared to the protonated thiol, oxidation of Cys is more prevalent at alkaline conditions. Oxidation of Cys, particularly when it leads to inter- or intramolecular disulfide formation, can be deleterious with respect to protein activity (see below).

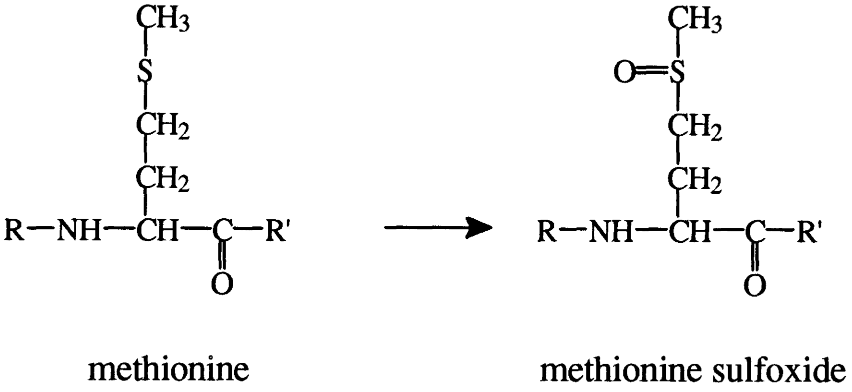


Figure 2.3 Oxidation of methionine residues.

2.2.6 β-Elimination of disulfide (cystine). Disulfide bonds, or cystines, are susceptible to β-elimination. This process occurs via a nucleophilic attack of a hydroxide ion on a carbon atom in a carbon-sulfur bond (Figure 2.4). The two new resulting residues, dehydroalanine and thiocysteine, are both relatively unstable (Volkin and Klibanov, 1989): the former may react with lysine, forming a lysinoalanine cross-link, and the latter decomposes to yield various thiol products such as hydrosulfide ions.

Since β-elimination is catalyzed by hydroxide ions, it is greatly accelerated under alkaline conditions. Because disulfides play a role in maintaining a protein's three-dimensional structure (Torchinski, 1974), their scission via β-elimination may have dire consequences for the bioactivity of therapeutic proteins. A further deleterious consequence of β-elimination is that the thiols which are generated may subsequently catalyze disulfide exchange.

2.2.7 Incorrect disulfide formation (disulfide scrambling). Disulfide bonds constitute an important structural motif in proteins. The formation of non-native, (incorrect) intra- or intermolecular disulfides may lead to drastic changes in protein structure and function (Torchinski, 1974). In addition, this dramatic change in protein structure may also contribute to other destabilization pathways such as adsorption and precipitation (non-covalent aggregation; Section 2.2.1).

Intra- or intermolecular disulfide scrambling can occur by various pathways, depending upon the conditions. For example, at acidic conditions disulfides can be cleaved by attack of a proton yielding a sulfenium ion which can subsequently react with a sulfur atom in another disulfide (Benesch and Benesch, 1958), as depicted in Figure 2.5. The formation of disulfides at neutral to alkaline conditions requires the presence of sulfide ion (Cecil and McPhee, 1959). This can be present on the protein itself, in the form of Cys residues; in this case the pathway of disulfide formation is thiol-disulfide interchange (Figure 2.6). Note that the reactive thiolate ion is conserved in this process. Alternatively, the catalytic ion can be of small molecular weight, for instance hydrosulfide ion which has been generated by a β-elimination of an intact disulfide. This scenario is thiol-catalyzed disulfide exchange, as illustrated in Figure 2.7.

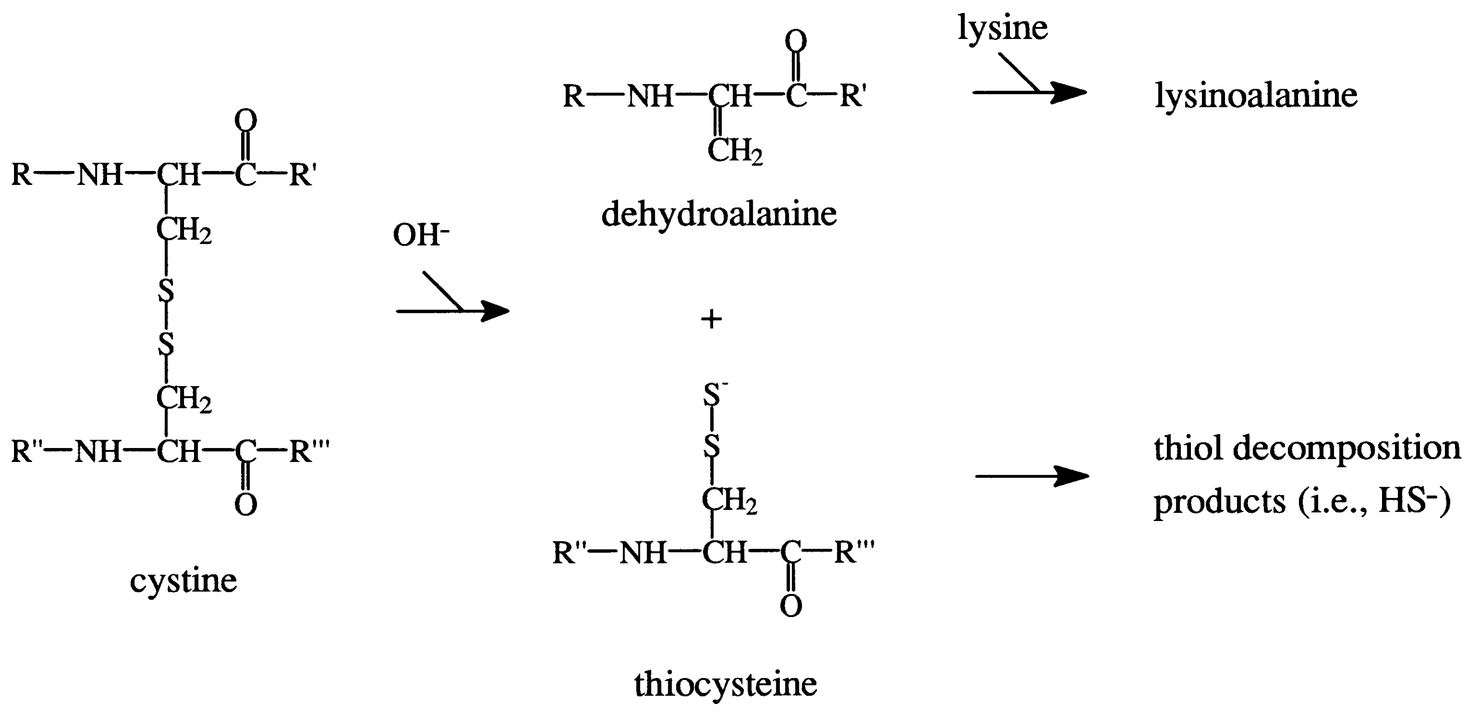


Figure 2.4 Mechanism of β-elimination of disulfide bonds (cystines) and formation of lysinoalanine cross-links.

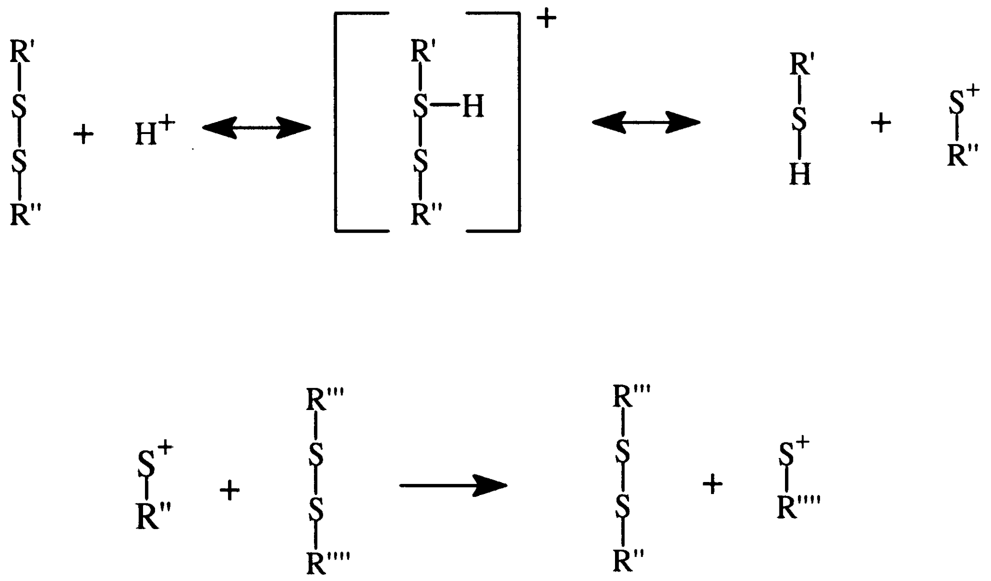


Figure 2.5 Disulfide interchange in acidic media.

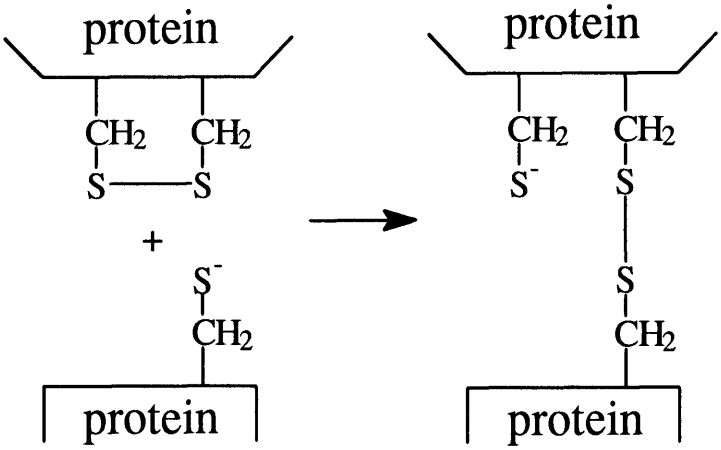


Figure 2.6 Thiol-disulfide interchange.

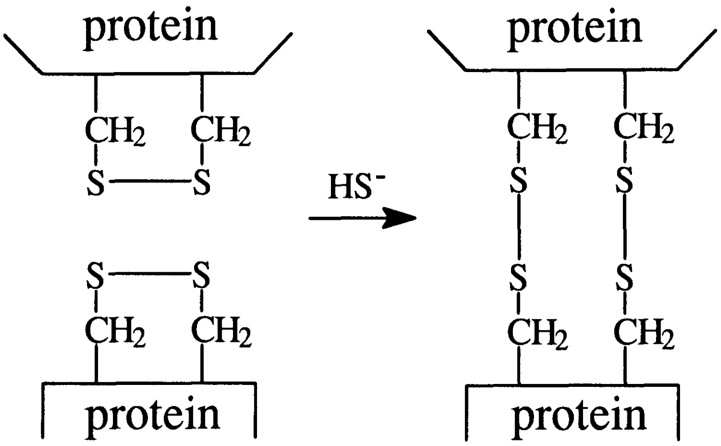


Figure 2.7 Thiol-catalyzed disulfide exchange.

Intermolecular disulfide-bonding has been shown to be responsible for the moisture-induced aggregation of a number of lyophilized proteins such albumin (Liu *et al.*, 1991; Jordan *et al.*, 1994; Costantino *et al.* 1995a) and tetanus toxoid (Schwendeman *et al.*, 1994, 1995c), suggesting it may be an important consideration in formulation of protein:polymer delivery depots.

2.2.8 The Maillard reaction. Another important, deleterious reaction involving proteins is the Maillard reaction (Figure 2.8). In this process, a Schiff base is formed as a result of the condensation of a Lys ε-amino group and the carbonyl group of a reducing sugar or aldehyde. This Schiff base is thought to undergo Amadori rearrangement to a more stable product (Hunt, 1985). Maillard-type reactions occur most readily in neutral to weakly alkaline conditions (Takahasi, 1977).

The Maillard reaction may be relevant to proteins and peptides loaded within polymer systems. Potential reactants include the reducing ends of various oligo- or polysaccharides (which may be candidates as matricies for sustained-release formulations) and the reducing ends of mono- or disaccharides which are commonly used as excipients in therapeutic protein formulations (Crowe *et al.*, 1987; Manning *et al.*, 1989; Cleland *et al.*, 1993; Hanson and Rouan, 1992; Timasheff, 1992). The Maillard reaction can occur in the solid state in the presence of moisture to therapeutic proteins, such as insulin (Schwartz and Lea, 1952). However, since most investigations of solid-state Maillard-type reactions have focused on hydrated food proteins (Duckworth, 1981), it is not certain whether, in general, the bioactivity of pharmaceuticals will be adversely affected.

2.2.9 Transamidation. Another deleterious, covalent process involving Lys is transamidation. In this reaction, the ε-amino group of Lys forms an isopeptide bond with the carbonyl group of either asparagine or glutamine (Figure 2.9), or dehydroalanine following β-elimination (Fig. 2.4) as discussed above. The cross-links thus formed may be either intra- or intermolecular.

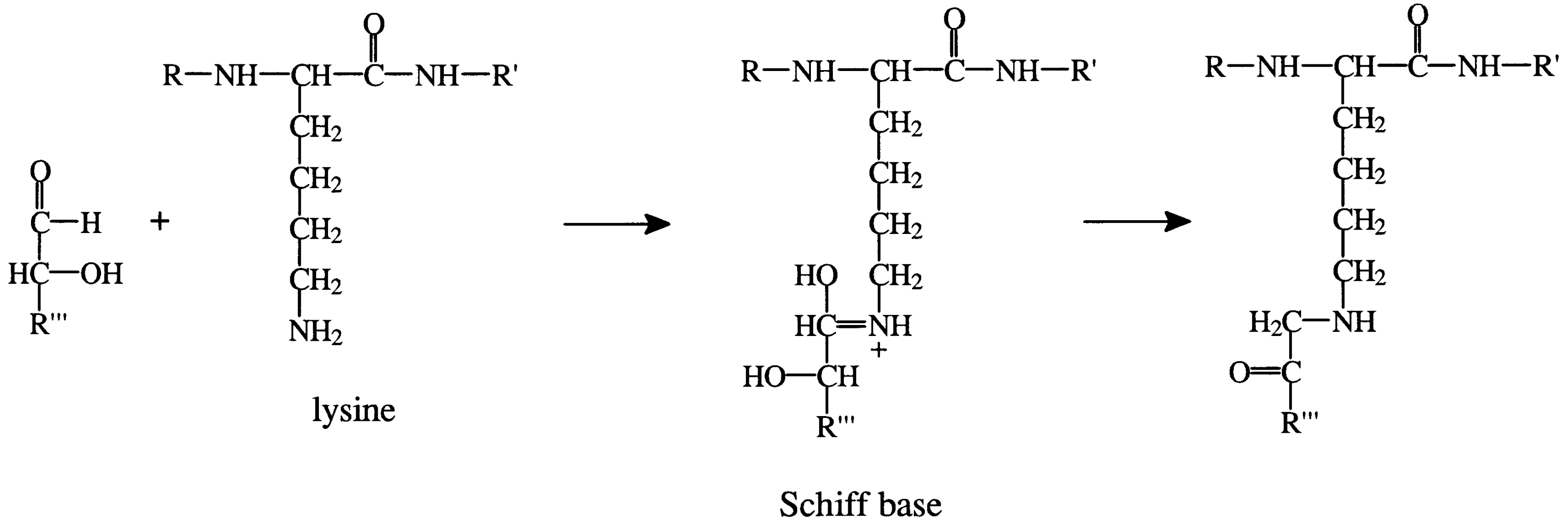


Figure 2.8 The Maillard reaction.

There have been various accounts of transamidation in solid proteins. Isopeptide bond formation may occur to solid enzymes suspended in organic solvents at elevated temperatures (Volkin *et al.*, 1991). Furthermore, it is proposed that intermolecular transamidation is responsible for the moisture-induced aggregation of the lyophilized therapeutic protein somatotropin (Hageman, 1988; Hageman *et al.*, 1992a) as well as for the aggregation of lyophilized ribonuclease A as evidenced by a loss of free Lys (as determined by amino acid analysis) in the insoluble, covalent non-disulfide-bonded aggregates (Townsend and DeLuca, 1988, 1991).

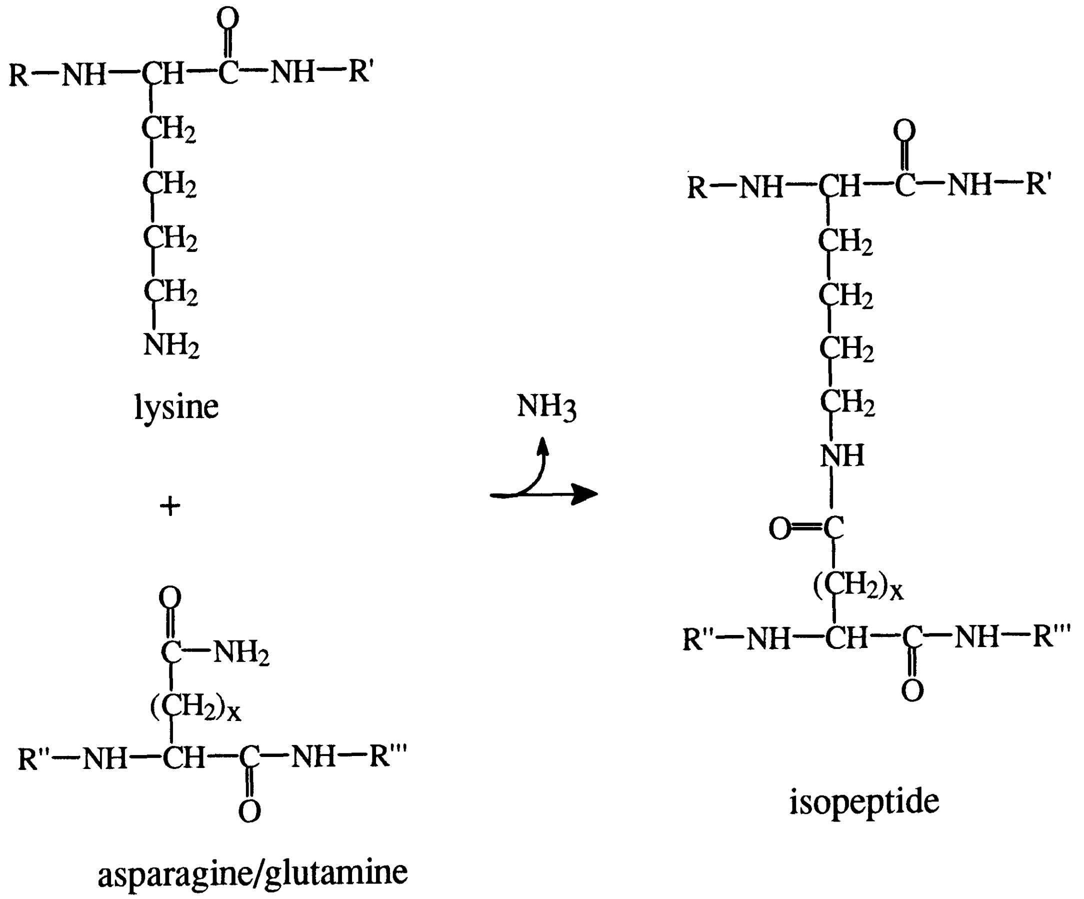


Figure 2.9 Isopeptide bond formation between lysine and asparagine (x=1) or glutamine (x=2) via transamidation.

2.3 Protein Stability Issues Related to Storage and Shelf Life of Proteins Formulated as Solids

Like any other pharmaceutical, therapeutic protein formulations must be designed to exhibit superior stability during storage. The FDA requires less than 10% deterioration over a two-year storage period (Cleland and Langer, 1994). To increase the stability of protein:polymer formulations, they are processed as solids, that is excess solvent is removed, typically by lyophilization.

2.3.1 Protein lyophilization. One method of obtaining a solid formulation is by lyophilization, or freeze-drying, which is commonly used to improve the shelf-life of biopharmaceuticals (Pikal, 1990a, 1990b; Franks, 1990, 1994; Franks *et al.*, 1991; Fox, 1995). However, the process of lyophilization may itself cause protein aggregation (Pikal, 1990b; Cleland *et al.*, 1993) and, in some circumstances, lyophilized therapeutic proteins may actually exhibit decreased stability compared to aqueous formulations (Hsu *et al.*, 1991).

2.3.2 Glass transition temperature (T_g). It has been proposed that the stability of lyophilized (or freeze-dried) proteins during storage is dependent upon the glass transition temperature (T_g). The reactivities of proteins in the rubbery state (above the T_g) are typically higher than in the glassy state due to increased molecular motions, as described in the literature (Franks *et al.*, 1991; Franks, 1994; Angell, 1995). As a rule of thumb, it is advantageous to store solid protein formulations at a temperature of at least 20°C below their glass transition (Franks, 1994) at low humidity (the presence of moisture dramatically reduces the T_g of proteins as discussed below).

Instead of controlling the storage temperature to ensure it is below T_g, one may also alter the protein formulation in order to raise its T_g. To this end, it has been proposed that the storage stability of protein formulations may be enhanced by incorporation of excipients with a higher T_g than the protein (Pikal, 1990b; Franks *et al.*, 1991; Hageman, 1992). One relationship which can be used to describe the T_g of protein:excipient systems is the Gordon-Taylor (G-T) equation (1952),

$$T_{g,\text{mixture}} = \frac{w_1 T_{g,1} + k w_2 T_{g,2}}{w_1 + k w_2}$$

where $T_{g,\text{ mixture}}$ is the observed glass transition for the mixture consisting of components 1 and 2 with their mass fractions denoted by w_1 and w_2 and their pure glass transition temperatures denoted by $T_{g,1}$ and $T_{g,2}$, and k is a constant.

2.3.3 Moisture content. Another critical factor in shelf stability of solid therapeutic proteins is the level of moisture. Water's role in the stability of solid proteins has been previously established as discussed in several reviews (Hageman, 1988, 1992; Ahlneck and Zografi, 1990; Costantino *et al.*, 1994b). The presence of water in solid protein formulations may be harmful for several reasons: (1) water increases protein molecular motions (the 'molecular lubricant' effect) which increases protein reactivity; (2) water is a reactant in many deleterious reactions (see below), and; (3) water is the mobile phase for reactants. This first (and perhaps most important) point, that water acts as a plasticizer, may be understood in terms of a significantly lowered T_g of the hydrated vs. dry formulation. (Ahlneck and Zografi, 1990; Franks *et al.*, 1991; Hageman, 1992; Franks, 1994; Angell, 1995). The T_g of hydrated protein formulations has been successfully described by the G-T equation (Kalichevsky *et al.*, 1993); note that $T_{g,\text{water}}$ = -139°C (Sugisaki *et al.*, 1968) , which is far lower than that of proteins.

Thus, another rule for proper storage conditions, besides keeping the temperature below the T_g, is to maintain low levels of moisture. The term "low" used herein usually refers to a water content near or below the theoretical monolayer coverage. However, it is not necessarily the case that the lower the moisture content the greater the stability. Hsu and co-workers (1991) have shown that for tissue-type plasminogen activator stored at 50°C, aggregation (increase in opalescence upon reconstitution) occurred more rapidly for the protein below monolayer water coverage compared to that at or above the monolayer coverage.

2.4 Stability Issues Related to *In Vivo* Delivery of Solid Protein Formulations

Ultimately, protein or peptide loaded within a polymer depot intended for sustained release (Pitt, 1990; Langer, 1993; Cleland and Langer, 1994) must exhibit bioactivity upon its delivery *in vivo*. However, deleterious processes may be induced by the microenvironment within the matrix during this period (Costantino *et al.*, 1994b; Schwendeman *et al.*, 1995a, 1995b). For example, there will be elevated temperature (37°C) in addition to the presence of a potentially harmful polymer interface and monomeric degradation products. Furthermore, the water content within the device will increase as water diffuses into the matrix. As a critical consequence, the protein will become increasingly hydrated which, in turn, may decrease its stability. The kinetics and ultimate level of hydration will depend upon the system, but may be quite dramatic.

2.4.1 Solid-phase protein aggregation. Whereas intramolecular processes may or may not affect the biological activity, protein cross-linking almost always leads to severely reduced biological activity (Manning *et al.*, 1989; Cleland *et al.*, 1993). Furthermore, the large size of protein aggregates leads to other problems, such as decreased solubility, slower release rate from the matrix due to diffusional limitations, and increased immunogenicity (Pinckard *et al.*, 1967; Moore and Leppert, 1980; Robbins *et al.*, 1987). Moreover, it has been suggested that due to the relatively high effective concentration in the solid state, intermolecular processes (i.e., aggregation) should be more prevalent than intramolecular processes (Hageman, 1992; Hageman *et al.*, 1992a).

Thus, aggregation is a serious obstacle to the delivery of protein from solid polymers. For example, the observed cross-linking of solid somatotropin at elevated temperature and humidity (Hageman, 1988; Hageman *et al.*, 1992a) may account for the incomplete release of this protein from a variety of polymeric matrices *in vitro*. These systems include porcine somatotropin in poly[lactic-*co*-glycolic acid] microspheres (Wyse *et al.*, 1989), and bovine somatotropin in various matrices including ethyl cellulose pellets (Sivaramakrishnan *et al.*, 1989), poly[ethylene-*co*-vinyl acetate] slabs (Sivaramakrishnan *et al.*, 1989) and polyanhydride matrices (Ron *et al.*, 1993).

2.4.2 Role of moisture on solid-phase protein aggregation. The critical role of water in protein structure, function, and stability has been well established (Hageman, 1988, 1992; Rupley *et al.*, 1983; Rupley and Careri 1991; Zaks, 1992). The factors that contribute to water's destabilizing role in solid protein aggregation were discussed above (Section 2.3.3). Perhaps the most important effect is that water increases protein flexibility, resulting in greater accessibility of reactive groups. This effect is pronounced at a hydration level between 25-38 g water/100 g protein, as described by Rupley *et al.* (1983) for solid lysozyme at room temperature. Below this level, mobility of bound water and protein internal motions are low; above it, the behavior approaches that observed at full hydration.

Increased protein flexibility with increasing moisture content may also be discussed in terms of lowering the T_g. For solid protein being hydrated within a polymeric implant, the T_g may fall below physiological temperature. For most proteins, this occurs at a hydration of approximately 20 g water/100 g dry protein (Kakivaya and Hoeve, 1975; Hageman, 1992; Kalichevsky *et al.*, 1993; Angell, 1995). The consequence (as discussed above) is that deleterious reactions are accelerated in the rubbery state above the T_g. Furthermore, it has been hypothesized (Franks *et al.*, 1991) that the kinetics of deleterious reactions for proteins in the rubbery state near the T_g are governed not by classical Arrhenius behavior but instead by the Williams-Landel-Ferry (WLF) (1955) relationship,

$$\ln k = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)}$$

where k is the rate constant, and C_1 and C_2 are constants. Since rate constants increase with $(T-T_g)$, as T_g is lowered by increasing water content, reaction rates increase at a given temperature (e.g., 37°C). The WLF theory has been successful in describing the kinetics of deleterious processes occurring to solid proteins (Roy *et al.*, 1991; Karmas *et al.*, 1992). For example, WLF behavior has been confirmed for the Maillard reaction occurring in hydrated food proteins at temperatures up to 10°C above their T_g (Karmas *et al.*, 1992). The WLF relationship may be useful in describing the temperature dependency of moisture-induced protein aggregation as well.

Increased susceptibility towards solid-phase aggregation with increasing hydration has been observed in several systems. For example, the solid-state thiol-disulfide interchange of bovine serum albumin (BSA) was markedly moisture-dependent (Liu *et al.*, 1991). In this investigation, solid protein of varying water content was prepared by mixing the lyophilized powder with aqueous buffer. The extent of aggregation (solubility loss) of these samples following a 24-h incubation at 37°C is depicted in Figure 2.10. With no added aqueous buffer, the extent of BSA aggregation was very low–less than 10%. As the water content was increased, aggregation became more rapid, reaching a maximum at about 40 g water/100 g dry protein, where over 90% solubility loss was observed. This hydration level is in the range where increased mobility effects are important, which probably accounts for the increased aggregation. At water contents above this level, the aggregation actually decreased. Similar behavior (bell-shaped aggregation vs. water content dependencies) was observed for β-lactalbumin, which also aggregated via thiol-disulfide interchange, and ovalbumin and glucose oxidase, both of which exhibited aggregation via non-covalent interactions (Liu *et al.*, 1991).

Other proteins have also exhibited higher rates of solid-phase aggregation with increasing water activity. For instance, somatotropin (bovine and human) undergoes a solid-phase aggregation which depends on the moisture level (Hageman, 1988; Hageman, 1992a). The initial aggregation rate for solid protein (lyophilized from pH 8.8), doubled from approximately 0.66% day^{-1} to 1.25% day^{-1} when the relative humidity was increased from 75% to 90% (Hageman *et al.*, 1992a) at 30°C. According to the sorption isotherm reported for bovine somatotropin (Hageman *et al.*, 1992b), these humidities correspond to equilibrium water contents of approximately 17 and 25 g/100g dry protein, respectively.

Similarly, the aggregation of ribonuclease A was influenced by the water content in the lyophilized powder. Protein lyophilized from pH 6.4 and incubated at 45°C exhibited approximately 20% aggregation (as determined by activity upon reconstitution) at a water content of about 2 g/100g dry protein following 120 days of incubation, whereas over a 50% loss ensued under these conditions at a water content of about 11 g/100g dry protein (Townsend and DeLuca, 1990).

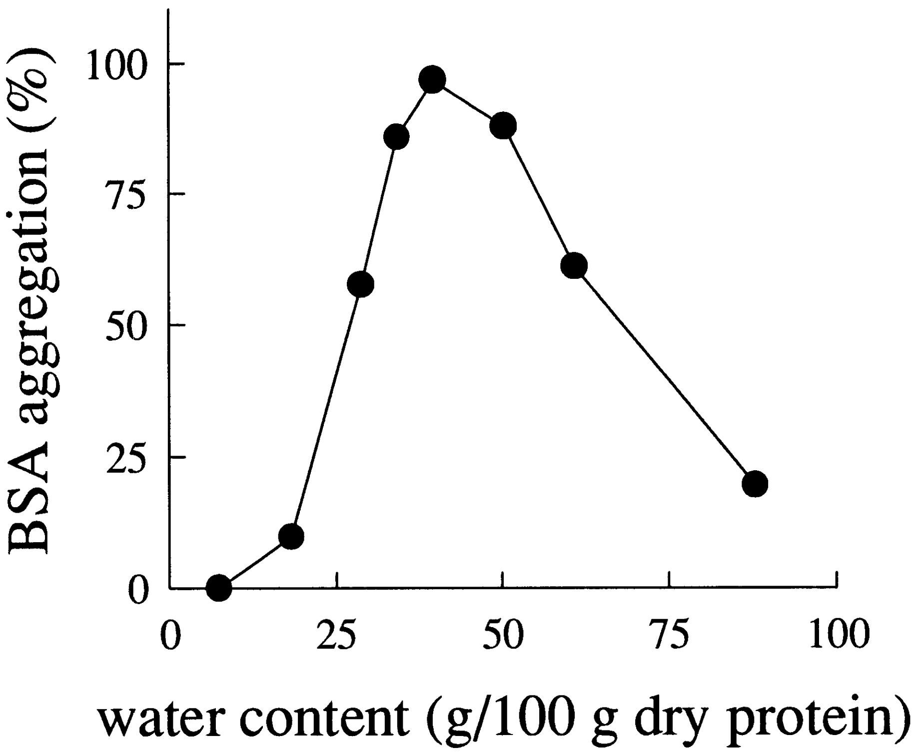


Figure 2.10 Effect of water content on solid-state aggregation of bovine serum albumin. Aggregation (i.e., solubility loss) was measured following a 24-h incubation at 37°C as a function of water content. Data adapted from Liu *et al.* (1991).

2.5 Rational Approaches for Stabilization of Proteins in the Solid Phase

Given the various molecular pathways that contribute to aggregation of solid proteins, rational approaches for stabilization can be developed. This strategy is more efficient (and intellectually satisfying) than a random search for conditions or agents to yield more stable solid protein formulations. The first approach is to specifically target the mechanisms involved. A second approach is to maintain the level of moisture activity in the protein solid at optimal levels. This may be simply achieved by storing the protein at optimal hydration levels, or, for the sustained-released device case, by choosing a microenvironment (i.e., polymer) that will ensure lower water activities. Another consequence of this approach is that one can also control the pH of the microenvironment. A third approach for stabilizing solid protein formulations is to increase the physical stability of the lyophilized protein. This will inhibit aggregation via hydrophobic interactions as well as via covalent pathways which may increase as proteins unfold.

2.5.1 Targeting the aggregation mechanism. One strategy for stabilizing proteins against solid-phase aggregation is to target the mechanism specifically responsible. As an illustration, the thiol-disulfide interchange (Fig. 2.6) of lyophilized albumin in the presence of moisture may be prevented by a variety of such approaches. Because aggregation requires free thiols, their elimination should arrest aggregation. Indeed, when the Cys^{34} residue of BSA was S-alkylated (using either iodoacetamide or iodoacetic acid as the alkylating agent), no aggregation occurred in the wetted lyophilized protein (Liu *et al.*, 1991). Thiol-disulfide interchange may also be prevented by controlling the pH; it is the thiolate ion, $-S^-$, rather than the protonated -SH group that is the reactive species (Fig. 2.6). Thus, adjusting the pH (prior to lyophilization) at lower levels should decrease rate of aggregation. This was verified experimentally for both bovine (Liu *et al.*, 1991) and human albumins (Costantino *et al.*, 1995a).

2.5.2 Controlling the activity of water. A general way to stabilize proteins against solid-state aggregation is to control the water content in the solid formulation. Because it profoundly influences solid-phase aggregation (Section 2.4.2), the water activity in the solid protein should be maintained at a judicious level. This level depends on the nature of the protein; in general, proteins maintained below their "monolayer" water coverage will exhibit superior solid-state stability (Hageman, 1988, 1992).

The control of moisture levels in proteins during delivery from solid systems is not straightforward. Depending upon the polymer matrix within which it is placed, proteins will experience significant hydration after the depot system has been implanted. However, in this case it is possible to control the microenvironment of the protein by changing the matrix. For example, water contents within the polymer bulk *in vitro* are as low as 1% for certain polyanhydrides (Shieh *et al.*, 1994) and up to 60% for poly[glycolic-*co*-lactic acid] (Shah *et al.*, 1992). Choice of matrix material will afford not only control of water activity within the depot, but also other important factors influencing aggregation, such as hydrophobicity and pH.

Solid proteins embedded within polymeric systems exhibit superior stabilities compared with their behavior under otherwise identical conditions in solution (Cleland and Langer, 1994; Ron *et al.*, 1993; Cohen *et al.*, 1991; Tabata *et al.*, 1993). This stability may be further enhanced by increasing the hydrophobicity of the polymer to limit the water content within the device or by reducing the thickness of the device, thereby allowing for more rapid dissolution and thus a shorter time of hydration. For example, Ron *et al.* (1993) have increased the cumulative *in vitro* release of bovine somatotropin from <50% to approximately 90% by changing the polymer from poly[1,3-bis(*p*-carboxyphenoxy)propane-*co*-sebacic anhydride] to a more hydrophobic one, poly[1,3-bis(*p*-carboxyphenoxy)hexane], under otherwise identical conditions. It should be noted, however, that exposure to hydrophobic surfaces may also promote denaturation and non-covalent aggregation, particularly for relatively hydrophobic proteins like the insulin monomer (Thurow and Geisen, 1984; Sluzky *et al.*, 1991).

2.5.3 Controlling the physical stability. Physical or conformational stability of proteins is often linked to protein aggregation. Loss of native structure may result in aggregation via hydrophobic interactions or increased rates of aggregation via other mechanisms due to increased exposure of reactive groups (Manning *et al.*, 1989; Volkin and Klibanov, 1989; Cleland *et al.*, 1993). It is therefore generally desirable to maintain the native protein structure in the solid state, especially considering that dehydration may induce structural rearrangements (see section 2.2.3).

Even if the lyophilized state is in the native conformation, unfolding may still occur under pharmaceutically relevant conditions. This may happen, for instance, if the temperature exceeds the melting temperature (T_m). For solid proteins, the T_m decreases significantly with increasing water content (Volkin *et al.*, 1991). Even when dry, proteins may unfold at the solid-void interface at elevated temperatures (Hsu *et al.*, 1995).

In aqueous solution, a protein's physical stability may be increased by addition of various agents, such as sugars, amino acids, and certain salts (Manning *et al.*, 1989; Cleland *et al.*, 1993; Hanson and Rouan, 1992; Timasheff, 1992). (The most ubiquitous mechanism of protection afforded by these agents is through their preferential exclusion from the protein surface (Timasheff, 1992).) However, not all of these agents are equally useful in stabilizing the folded structure during incubation in the solid state. For example, Carpenter and co-workers (Arakawa *et al.*, 1991; Carpenter and Crowe, 1989; Carpenter *et al.*, 1991; Prestrelski *et al.*, 1993a, 1993b) have demonstrated via FTIR that only certain agents, such as saccharides, are strong lyoprotectants and it has been hypothesized that these agents stabilize the native structure through hydrogen bonding (substituting for water). It should be noted here that an alternate hypothesis to explain the stabilization afforded by these lyoprotectants is that they may increase the glass transition temperature of the lyophilized powder (Franks *et al.*, 1991; Franks, 1994; Hatley and Franks, 1991; Chang and Randall, 1992).

Regardless of the mechanism responsible, it is clear that certain additives, in particular sugars and other polyols, impart significant physical stability to lyophilized proteins. A number of reports have demonstrated that these agents can be used to protect proteins against aggregation not only during lyophilization but also during storage in the dry state. For instance, the addition of mannitol inhibited the solid-phase aggregation of dry human somatotropin (lyophilized from pH 7.4) at either 25°C or 40°C (Pikal *et al.*, 1991). Another example is ribonuclease for which it was reported that excipients such as sucrose and Ficoll 70 (a polymer with sucrose units) afforded dramatic protection against aggregation during solid-phase incubation under various conditions (Townsend and DeLuca, 1988).

Such excipients may also enhance the stability of solid proteins embedded within polymeric matrices. Incorporation of sucrose within a sustained-release formulation of bovine somatotropin in poly[ethylene-*co*-vinyl acetate] increased cumulative release of the protein *in vitro* at 37°C from approximately 13% in the absence of sucrose to over 40% for a formulation containing a protein-to-sucrose weight ratio of 1:2 (Sivaramakrishnan *et* *al.*, 1989). Similarly, it has been reported that improved release of this protein from polyanhydride matrices was achieved upon addition of sucrose (Ron *et al.*, 1993).

2.6 Concluding Remarks

In order to successfully employ proteins as pharmaceuticals, it is essential to understand mechanistically the stability issues relevant to their formulation and delivery. Various deleterious processes may occur in solid protein formulations, thereby diminishing their therapeutic value. Of these, moisture-induced aggregation (by various covalent and non-covalent pathways) is perhaps the most serious problem. By using rational approaches, however, one can protect proteins against this, and other deleterious processes.

CHAPTER 3

EXPERIMENTAL APPROACH

3.1 Study of Solid-Phase Protein Aggregation

The study of solid protein aggregation under pharmaceutically relevant conditions involves several steps: formulate protein as solid; incubate it under pharmaceutically relevant conditions; reconstitute into an aqueous system (comparable to the *in vivo* environment) and conduct biochemical characterization in order to elucidate deleterious processes. In this chapter, the experimental approach will be presented, along with specific materials and methods employed.

3.1.1 Selecting pharmaceutically relevant experimental conditions. In order to investigate stability of solid proteins under pharmaceutically relevant conditions, it is necessary to create an environment that mimics that of solid protein administered *in vivo* or represents the most extreme conditions experienced during storage. For example, protein suspended within a polymeric matrix would experience elevated temperature (i.e., 37°C) and water activity during its use. Thus, a methodology to reproducibly simulate this environment needs to be developed in order to test protein stability under these conditions.

To control the level of moisture, the most straightforward method would be to add various amounts of water directly to the lyophilized protein powder and incubate at the desired temperature. However, uniformly mixing small amounts of protein and water may be difficult, and this level of protein hydration may not be the equilibrium state (desorption may ensue). Another approach is to incubate the protein at the desired temperature in a controlled-humidity chamber. The constant level of humidity is achieved by the presence of a saturated solution of an inorganic salt within the chamber (Youngs, 1967; Greenspan, 1977). This methodology leads to reproducible water activities once the water sorption has reached equilibrium and also obviates the problem of mixing the protein and water.

If observed aggregation rates are too slow to measure experimentally under pharmaceutically relevant conditions, it is necessary to alter the conditions in order to accelerate aggregation. For example, the lyophilization conditions (for example, pH of the aqueous solution prior to freezing or the presence of destabilizing agents) can be manipulated in such a way as to increase aggregation. However this approach may significantly affect the chemical or physical state of the protein. Hence, the preferred approach is to raise the temperature. This strategy of examining stability under accelerated storage conditions (elevated temperature) is commonly used in the pharmaceutical industry; however, the FDA ultimately requires real-time data for shelf-life stability determinations (Cleland and Langer, 1994).

One must exercise caution in extrapolating stability data obtained at accelerated storage conditions to less extreme ones that are pharmaceutically relevant *in vivo* (37°C) or for *in vitro* shelf storage (4°C). Because multiple pathways with different activation energies may exist, raising the temperature may change the dominant mode of protein deterioration. Another important factor to consider is that the protein conformation may change (unfolding may occur) at elevated temperatures which may alter the activation energies of degradation pathways. If the temperature is above the glass transition temperature (T_g), the solid formulation will change its physical state from a glassy to a viscoelastic, or rubbery one (Angell, 1995) with implications for the rates of solid-state reactions (see Section 2.3.2). In this case, kinetics may exhibit WLF, and not Arhennius behavior. These considerations preclude the extrapolation of accelerated stability data from above T_g to below this temperature. If these difficulties are taken into account, then stability studies at accelerated storage conditions can be useful tool for evaluating pharmaceutical protein formulations.

3.1.2 Elucidating mechanisms of solid-phase protein aggregation. Once the conditions have been chosen, the next step is to incubate the protein and uncover possible deleterious mechanisms. In this case, following incubation, one must reconstitute the protein in aqueous media—physiological conditions are most appropriate. Based on the amount of protein dissolved against an unincubated standard, one can determine how much aggregation, or intramolecular links leading to solubility loss, has occurred. In addition, one can investigate the nature of soluble oligomeric species by size-dependent analytical techniques such as size-exclusion chromatography.

To investigate the nature of the cross-links responsible for the loss of solubility, one can attempt to dissolve the aggregates in various aqueous solutions. If the aggregates are held together by non-covalent forces (hydrophobic interactions), the aggregates should dissolve in high concentrations of denaturing agents such as urea and guanidine hydrochloride. If the aggregates are held together by disulfide bonds, the addition of a disulfide reducing agent, such as dithiothreitol, should be sufficient to dissolve the aggregates. If neither a denaturing nor a thiol reducing agent dissolves the aggregates, than another covalent mechanism is responsible for the aggregation. In this case, the elucidation of the aggregation is more difficult. One possibility is the lysinoalanine cross-link, which can be identified upon acid hydrolysis and amino acid analysis of the aggregates. Others have implicated lysine as a member of a cross-link in transamidation or other pathways; loss of lysine can also be followed via acid hydrolysis and amino acid analysis or perhaps by the liberation of NH_3. The aggregation picture becomes more complex when more than one mechanism of cross-linking is occurring, which may be a common case for solid pharmaceutical proteins. (Liu *et al.*, 1991; Costantino *et al.*, 1994a, 1994b; Schwendeman *et al.*, 1993).

3.2 Materials

3.2.1 Proteins. Recombinant human albumin (rHA: M_w=66 kDa) was a gift of Delta Biotechnology Ltd. (Nottingham, England). Bovine Zn-insulin (M_w=5.5 kDa) was purchased from Sigma Chemical Co. (St. Louis, MO). Human Zn-free insulin was a generous gift from Eli Lilly & Co. (Indianapolis, IN). Tetanus toxoid (TT: M_w=150 kDa) was obtained from the Massachusetts Public Health Biologic Laboratory (MPHBL, Boston, MA) in two lots: PS-21, a purified (85% monomeric) preparation exhibiting an activity of 1663 Lf/ml [3.9 μg/Lf] and LP-943P (420 Lf/ml [4.0 μg/Lf]), a partially purified (50% monomeric) preparation used for water-sorption measurements. Diphtheria toxoid (DT: M_w=58 kDa) was also provided from MPHBL (lot DCP-54, 540 Lf/ml [3.1 μg/Lf]).

3.2.2 Other chemicals. Carboxymethyl-dextran (M_w=500 kDa), dextran (M_w=590 kDa; M_n=350 kDa), and poly[ethylene glycol] (20 kDa), were purchased from Polysciences, Inc. (Warrington, PA). D-Glucamine and guanidine hydrochloride were from Fluka Chemical (Ronkonkoma, NY). Anhydrous dimethylsulfoxide (DMSO), glycolic acid, D-lactic acid (lithium salt), L-lactic acid (85% aqueous solution), and propionic acid (Na salt) were from Aldrich Chemical Co. (Milwaukee, WI). α-*N*-Acetyl-L-lysine methyl ester, L-alanine, diethylaminoethyl-dextran (M_w=500 kD), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), dithioerythritol (DTE), D-glucaric acid (potassium salt), D-gluconic acid (50% aqueous solution), D-glucosaminic acid, D-glucose diethyl mercaptal, *n*-octanoic acid, L-serine, sodium cyanoborohydride (CBH), D-sorbitol, succinic anhydride, sucrose, D-trehalose (reduced metal ion content), 2,4,6-trinitrobenzene sulfonate (TNBS, 5% aqueous solution), and urea were from Sigma Chemical Co. The KBr used for preparing solid samples for IR spectroscopy was from Spectra-Tech (Shelton, CT). Glycine was obtained from Mallinckrodt (Paris, KY). All other chemicals were analytical grade or purer and obtained from commercial suppliers.

3.3 Methods

3.3.1 Preparation of rHA. rHA was provided as a 20-25% (w/w) aqueous solution containing 145 mM sodium chloride and 16 mM sodium octanoate, pH 7.0 ± 0.3. To remove the octanoate we employed a modified charcoal treatment (Chen, 1967). The rHA solution was diluted to 10 mg/ml with water containing 10 mg/ml of activated charcoal. The pH was then adjusted to pH 3.0 with 1 M HCl, and the mixture was stirred in an ice-water bath for 1 h. Charcoal was removed by centrifugation for 20 min at 20,000x*g*. To remove salts, the supernatant was dialyzed (12 kDa cutoff) at 4°C for 24 h with three changes of 0.1 mM sodium phosphate at pH 7.3. Prior to lyophilization, this dialyzate was diluted with water to 1 mg/ml protein (unless otherwise stated). The desired excipient was added to the rHA solution at this time, followed by pH adjustment to pH 7.3 with 0.1 M HCl or NaOH. This protein liquid was then freeze dried (see Section 3.3.4). The lyophilized rHA had 0.78-0.88 mol-equivalents of free thiols (see Section 3.3.9).

3.3.2 Preparation of insulin. Bovine Zn-free K-insulin was prepared as follows. The Zn-insulin (100 mg) was dissolved in 15 ml of 0.04 M HCl, and the solution was adjusted to pH 6.0 by addition of approximately 13 ml of 0.04 M KOH containing 10 mM EDTA. The resulting insulin precipitate was collected by centrifugation at 5000x*g* for 10 min. This procedure was repeated twice before the insulin pellet was dissolved at pH 3.0 (acidic pH was attained with HCl) to the desired concentration by stirring for 20 min. The solution was then adjusted to pH 7.3 with KOH, filtered using a Nalgene filter (0.2 µm pore size) and lyophilized.

Bovine Zn-insulin was dissolved in water by stirring either for 20 min at pH 3.0 and room temperature or for several hours at pH 10 and 37°C. Whereas Zn-insulin is easily solubilized at pH 3.0, its dissolution in alkaline medium is very slow; even at concentrations as low as 0.05 mg/ml, there was visible turbidity in the solution after 2 h of stirring at 37°C. Therefore, all aqueous insulin samples were clarified by filtration (using the Nalgene filter), and the final pH prior to lyophilization was adjusted to 7.3 (with HCl or NaOH), unless otherwise stated. Insulin dissolved at 0.05 mg/ml by stirring at 37°C and pH 10 for 2 h, and finally adjusted to pH 7.3 prior to lyophilization, is referred to in the text as 'rapidly aggregating' insulin.

3.3.3 Preparation of TT and DT. TT was provided as an aqueous formulation. Prior to lyophilization, all samples were dialyzed (12 kDa cutoff) against 1 mM sodium phosphate, pH 7.3 (unless otherwise indicated) and diluted to approximately 1 mg/ml. Note that for some experiments, a higher buffer concentration (10 mM) was used, an indicated. TT was routinely lyophilized in 0.25-ml aliquots. Preparation of DT was conducted similarly.

Chemical modification of TT was accomplished as follows. The reduction of disulfides was carried out in aqueous solution consisting of 140 mM DTT, 0.29 M Tris buffer, pH 8.6, for 2 h at 37°C. To protect free amino groups, TT (or DT) was treated with succinic anhydride (2.4:1 w/w) at pH 7 (Hollecker, 1989). The reduction of TT with 1 mg/ml CBH was conducted at pH 7 and 37°C for 1 h as previously described (Dottavio-Martin and Ravel, 1978), except that no formaldehyde was present. Following all modifications, any excess DTT, succinic anhydride, or CBH was removed by desalting of a Sephadex G-50 column equilibrated with PB (100 mM sodium phosphate pH 7.3, 1 mM EDTA) or by dialysis.

3.3.4 Lyophilization. All samples were frozen rapidly in liquid nitrogen either in 2- or 5-ml vials or in 25 to 100-ml round bottom flasks, depending on the volume. These were then placed on a Labconco Freeze Drier 8 (Kansas City, MO) operating at a pressure of approximately 10 μm of Hg and a condenser temperature of -50°C. Typically, samples were removed after 48 h. Average water content of lyophilized proteins were as follows: rHA, 10.1±1.7 g/100 g dry protein; insulin, 5.3±0.9 g/100 g protein; and TT, 5 g/100g (see Section 3.3.12)

3.3.5 Rotary evaporation. An aqueous solution of rHA (10 mg/ml, pH 7.3) was rotary evaporated for 2 h on a Büchi 011 Rotavapor at 30°C with vacuum provided by an aspirator. The rotary-evaporated rHA had 0.80±0.04 mol-equivalents of free thiols and a water content of 2.5±0.4 g/100 g protein.

3.3.6 Spray drying. rHA was spray dried was on a Büchi Model 190 laboratory-scale apparatus in a manner similar to that reported previously (Mumenthaler *et al.*, 1994) Atomization of the aqueous protein (flow rate of 5 mg/ml of a solution consisting of 10 mg/ml, pH 7.3) was accomplished by means of a pressurized air stream flowing at 600 l/h. The drying air temperature was approximately 90°C. The spray-dried rHA had 0.77±0.03 mol-equivalents of free thiols and a water content of 4.0±1.2 g/100 g protein.

3.3.7 Acetone precipitation. rHA solution (10 mg/ml, pH 7.3) was stirred while incubated in a dry ice:acetone bath and cold acetone was added until the protein precipitated (Englard and Seifter, 1990). To remove any residual organic solvent, samples were placed under vacuum (10 μ torr) for several hours. The acetone-precipitated rHA had 0.72±0.03 mol-equivalents of free thiols and a water content of 4.8±0.4 g/100 g protein.

3.3.8 Determination of octanoate. In order to verify its removal from rHA during the charcoal treatment (Section 3.3.1), octanoate was extracted by mixing 0.5 ml of an aqueous solution consisting of 10 mg of lyophilized protein, 100 mM citric acid pH 3.0, with 2.5 ml of isopropyl alcohol:isooctante:1 M H_2SO_4 (40:10:1) and stirring the mixture for 15 min (Chen, 1967). The octanoate was then measured using gas chromatography (10-m HP-5 capillary column coated with 5% phenyl-/95% methyl-silicone gum (Hewlett Packard)).

3.3.9 Determination of free thiols. Free thiols were measured using Ellman's reagent, DTNB, which reacts rapidly and quantitatively with the thiolate ion to yield the 2-nitro-5-thiobenzoate anion (TNB^{2-}) which has the absorption maximum at 412 nm (Riddles *et al.*, 1983). Proteins were dissolved (typically at 2 mg/ml) in PB and then filtered through a 0.22 μm Millex-GV filter. Varying amounts of the protein solution were added to the reaction mixture which had a final DTNB concentration of 0.3 mM (in PB), and the free thiol concentration was calculated from the absorbance of the reaction mixture at 412 nm (Riddles *et al.* 1983). For insulin, to determine whether the free thiols had a low molecular weight (e.g., hydrosulfide ion) or were protein-bound, the protein was concentrated approximately 3-fold by loading the sample on an Amicon Centricon-3 microconcentrator (with 3000 Da molecular weight cutoff) and centrifuging the microconcentrator for 1 h at 5000xg. The free thiol content of the filtrate was compared with that of the retentate (which retained the concentrated insulin) and with that of the unfiltered sample.

3.3.10 Determination of free amino groups. The number of accessible free amino groups for TT and DT was measured using TNBS, as described previously in the literature (Habeeb, 1966a). α-*N*-Acetyl-L-lysine methyl ester was used as a standard.

3.3.11 Quasielastic light scattering. Quasielastic light scattering is a procedure whereby information may be obtained regarding mean particle diameter and the distribution of particle sizes in solution. This technique has been utilized in determining particle size distribution of insulin during aggregation in agitated aqueous solution (Dathe *et al.*, 1990; Sluzky, 1992; Sluzky *et al.*, 1991, 1992).

The light scattering apparatus consisted of a Lexel argon-ion laser, a 128-channel digital correlator and signal processor (Brookhaven Instruments, Holtsville, NY), and a microcomputer that was used for measurement control and data acquisition. A wavelength of 488 nm and a scattering angle of 90° were used for all measurements. The microcomputer was also employed to determine size-distribution function from the experimentally determined autocorrelation function using inverse Laplace transforms algorithms (both non-negatively-constrained least squares and CONTIN) (Sluzky, 1992). In buffered aqueous solution, insulin monomers and dimers both exhibit a hydrodynamic diameter of about 1-2 nm, and hexamers exhibit a hydrodynamic diameter of about 5 nm (Sluzky *et al.*, 1991; Sluzky, 1992).

3.3.12 Determinations of water content and water sorption isotherms. Measurement of protein-bound water was conducted by Karl Fischer titration using hydranal-titrant 5 and hydranal solvent (both from Crescent Chemical Co. (Hauppauge, NY)) with a Mettler DL18 autotitrator (Toledo, OH). This method has been routinely used to measure the water content of powders (Conners, 1988). The water content of dry (unincubated) samples was measured by direct application of the powder (5-10 mg) into the Fisher titrator.

Wetted (incubated) samples (0.5-4 mg) were stored at various relative humidities until equilibrium uptake of water by the lyophilized powder was achieved and no additional water was absorbed (for rHA, 24 h at 37°C; for insulin, 7 days at 25°C or 6 h at

50°C; for tetanus toxoid, 14 days at 37°C). Such rapid absorption of water has been observed for other proteins (Hageman *et al.*, 1992b). A constant relative humidity (rh) was achieved by the use of desiccators containing saturated salt solutions (Youngs, 1967; Greenspan, 1977) and depended upon the inorganic salt and the temperature (Table 3.1). Following storage, 1.0 ml of anhydrous dimethylsulfoxide (Aldrich Chemical Co.) was added to each 2-mg sample. This solution was then sonicated for 30 s (Ultrasonic Cleaner Model 250, RAI Research) and injected into the Fischer titrator. In this case, it was necessary to subtract out the water which was present in the DMSO (no sample added). All water contents are reported in g/100 g dry solid and represent the mean and standard error of at least three separate determinations.

3.3.13 Modeling of water sorption isotherms. The water vapor sorption isotherm for rapidly aggregating insulin was modeled using the Brunauer-Emmett-Teller (BET) (1938) gas adsorption equation:

$$M = \frac{a M_0 C}{(1-a)(1-a+Ca)}$$

where M is the equilibrium moisture content of the protein (in g water/100 g dry protein), a is the activity of the sorbed water (which is equal to rh/100), C is a constant related to the heat of absorption of the monolayer, and M_0 represents the monolayer coverage of water (in g of water per 100 g dry protein). The BET equation can be used to determine M_0 using the equation $M_0 = 1/(S+I)$, where S is the slope and I is the y-intercept of the plot of a vs. $a/\{(1-a)M\}$ (known as the BET plot). The BET model has been used extensively to describe the water vapor sorption isotherm and monolayer water sorption for proteins (Schnepf, 1989; Hsu *et al.*, 1991; Hageman *et al.*, 1992b).

3.3.14 Study of moisture-induced aggregation of solid proteins. Lyophilized, spray dried, and rotary evaporated rHA formulations (2 mg) were incubated at standard conditions of 37°C and 96% rh, unless otherwise stated. Constant rh was attained by use of a saturated salt solution within a desiccator (see Section 3.3.12). A septum port on the desiccator lid allowed for on-line measurements of temperature and rh with a VWR digital

Table 3.1

Humidity control using aqueous saturated salt solutions[a]

	Relative humidity (%) achieved at a temperature of:			
Salt	25°C	37°C	45°C	50°C
LiCl	11	11	11	11
$MgCl_2$	33	32	31	31
NaBr	58	54	52	51
$NaNO_2$	64	62	—[b]	—
KI	69	67	65	65
$SrCl_2$	71	67	—	—
$NaNO_3$	74	72	70	69
NaCl	75	75	75	74
$(NH_4)_2SO_4$	81	80	80	79
KCl	84	83	82	81
K_2CrO_4	87	86	—	—
KNO_3	94	90	87	85
$BaCl_2$	90	89	89	89
K_2SO_4	97	96	96	96

[a] All data taken from Youngs (1967) and Greenspan (1977).

[b] Not recommended for controlling relative humidity at this temperature.

hygrometer/thermometer. Other humidities (all at 37°C) used were 11%, 75% and 90%. (For description of the saturated salt solutions used for these and other rh, see Table 3.1.) In order to study the stability of rHA at water contents above that achieved at 96% relative humidity, water was directly added to lyophilized rHA (mixed with a stainless steel spatula), followed by incubation at 96% rh. Aggregation was determined by comparing protein concentrations (see Section 3.3.15) of incubated and unincubated samples following reconstitution. All data are presented as the mean and error of three independent incubations.

Following incubation, rHA samples were reconstituted (1 mg/ml) in phosphate-buffered saline (PBS: 150 mM NaCl and 5 mM sodium phosphate at pH 7.3) by stirring for 2 h at 37°C. Standards used for protein determinations were also treated in this manner, except they were not incubated in the constant-humidity chamber. To elucidate the mechanism of aggregation, samples were reconstituted in PBS containing various denaturing and/or reducing agents, as described, and stirred for only 30 min (at 37°C) to prevent precipitation upon extended exposure to such agents.

Investigation of the solid-state stability of insulin was conducted in a similar manner to that described above for rHA above, except that samples (1.0 mg) were weighed into 10-ml beakers which were then incubated at standard conditions of 96% rh and 50°C (unless otherwise stated) and reconstituted at 0.2 mg/ml protein in PBS. Tetanus toxoid was studied in a likewise manner, except that samples (0.25 mg) were incubated at standard conditions of 86% rh and 37°C (unless otherwise stated) and reconstituted at 0.5 mg/ml protein in PB.

3.3.15 Protein assays. Protein concentrations were measured by the bicinchoninic acid method (Pierce Chemical Co.), the Bio-Rad method (Bio-Rad Laboratories) and, in cases where there was interference with these assays, absorbance at 280 nm (Boolag and Edelstein, 1991). For all assays, unincubated protein from the same batch was used as a standard. Prior to conducting protein assays, all samples were filtered through a Millex-GV filter (Millipore) of pore size 0.22 μm in order to remove insoluble materials. All spectrophotometric measurements were conducted in a Hitachi U-3110 spectrophotometer.

3.3.16 Size-exclusion chromatography. The oligomeric composition of the soluble rHA fraction was analyzed by size-exclusion chromatography using FPLC (Pharmacia fast liquid chromatography system) with a Superose 12 gel filtration column (Pharmacia). The void and total column volumes were 7.9 and 20.0 ml, respectively. The mobile phase consisted of aqueous PBS with 0.02% sodium azide, and the flow rate was 1.0 ml/min. Lyophilized rHA was dissolved at 5 mg/ml in this buffer, and typically 0.1 ml was injected into the column. All samples (including unincubated protein) were filtered (Millex-GV filter (Millipore) with a pore size of 0.22 μm) prior to their loading. Peaks were integrated by the LCC control unit (Pharmacia) and the results checked manually.

3.3.17 Fourier-transform infrared (FTIR)spectroscopy. One method to obtain structural information on both solid and solution protein samples is Fourier-transform infrared (FTIR) spectroscopy. Two spectral regions, the amide I (1720-1600 cm^{-1}) and the amide III (1335-1215 cm^{-1}), are particularly sensitive to protein conformational changes and are thus suitable for structural investigation (Susi, 1969; Susi and Byler, 1986; Jakobsen and Wasacz, 1987; Wasacz *et al.*, 1987; Surewicz and Mantsch, 1988). Furthermore, a number of studies have specifically employed this strategy to probe the conformation of albumin (Jakobsen *et al.*, 1986; Kaiden *et al.*, 1987; Kato *et al.*, 1987; Wasacz *et al.* 1987; Fu *et al.*, 1994). Therefore, this technique was a logical choice for comparison of the relative amounts of elements of secondary structure between dried rHA and aqueous (native) protein and between various dried rHA formulations.

FTIR was conducted as described previously (Griebenow and Klibanov, 1995) with a Nicolet Magna-IR 550 Spectrophotometer (Madison, WI) equipped with a MCT-B liquid N_2-cooled detector, germanium beamsplitter on KBr substrate (7400-350 cm^{-1}), and a high intensity air-cooled mid-IR Ever-Glo source (9600-50cm^{-1}). Control of the instrument as well as collection and primary analysis of data were accomplished using Omnic 1.2 software (Nicolet) run on a PC. During all runs, the optical bench was purged with dry gaseous N_2 to reduce interfering water vapor IR absorption. For each run, a total of 256 scans at a resolution of 2 cm^{-1} using Happ-Genzel apodization were averaged.

The data measurement and correction have been thoroughly described in the literature (Singh and Fuller, 1991; Singh *et al.*, 1992; Griebenow and Klibanov, 1995). Solution spectra were measured at concentrations of 10 and 100 mg/ml for TT and rHA, respectively, using a 15-μm spacer in a Spectra-Tech liquid cell equipped with CaF_2 windows. Powders were measured at concentrations of 0.5-2 mg/200 mg KBr in pellet pressed using a Spectra-Tech Macro-Micro KBr Die Kit and a Carver (Wabash, IN) 12-ton hydraulic press. This methodology does not introduce any artifacual structural changes (Prestrelski *et al.*, 1993a, 1993b). All liquid and solid samples were measured at least five times. If necessary, a water and/or excipient spectrum was used for subtraction, thus yielding the protein spectrum (Griebenow and Klibanov, 1995; Costantino *et al.*, 1995b).

Wavenumbers of vibrational peaks were located using either second derivitization (for rHA) or Gaussian self-deconvolution (for TT) of the original (non-smoothed) spectrum. Using this information as a starting point, Gaussian deconvolution was performed on the IR spectra in the Amide III region, revealing peak wavenumbers and their relative peak areas. (This approach was problematic in the amide I region of the FTIR spectra due to strong band-overlapping, and the large signal for water.) These peaks were then assigned to various elements of secondary structure (α-helix, extended chain, random coil/turn) according to previous studies (Singh *et al.* 1993; Fu *et al.*, 1994; Griebenow and Klibanov, 1995).

CHAPTER 4

MOISTURE-INDUCED AGGREGATION OF SOLID RECOMBINANT HUMAN ALBUMIN

4.1 Motivation and Overview

The successful utilization of proteins and peptides as pharmaceuticals depends upon not only their production in biologically active form but also their delivery as stable formulations. Therapeutic proteins are often formulated as solids and in some cases are suspended within polymeric matrices intended for controlled release (Pitt, 1990; Langer, 1993; Cleland and Langer, 1994). However, in a number of instances solid proteins may undergo moisture-induced aggregation (see Chapter 2).

For example, it has been reported that lyophilized bovine serum albumin (BSA), a commonly used model protein, experienced a loss of solubility as a result of cross-linking via thiol-disulfide interchange when exposed to moisture at 37°C (Liu *et al.*, 1991). It was reported that co-lyophilization (50 wt%, dry basis) with a number of water soluble polymeric excipients of varying functionalities, such as poly[ethylene glycol], dextran, diethylaminoethyl (DEAE)-dextran and carboxymethyl (CM)-cellulose, all resulted in significant stabilization towards protein aggregation in the wetted powder (Liu *et al.*, 1991). It was hypothesized in this case that stabilization was imparted simply by a "dilution" effect; at a given water content the presence of the polymeric excipients resulted in decreased protein-protein contacts and hence reduced aggregation. This hypothesis was also consistent with the observation that the extent of aggregation (without excipient) decreased as the protein was diluted with additional water beyond approximately 40 g water/100 g dry lyophilized powder (Liu *et al.*, 1991).

Herein, the solid-state stability of a closely related protein, recombinant human albumin (rHA), will be considered. rHA is a logical model for study since it is a pharmaceutically useful protein (Peters, 1985) and a potential agent for the sustained delivery of various low-molecular-weight drugs which bind to it (He and Carter, 1992; Geisow, 1992). Initially, the kinetics and mechanism of rHA's moisture-induced aggregation were studied in the absence of excipients. Subsequently, rHA stability was tested in the presence of various polymeric and monomeric excipients. Despite their common use in stabilizing proteins during lyophilization or when stored in aqueous solution (Timasheff, 1992; Cleland *et al.*, 1993), few investigations have examined the effects such excipients may have upon the stability of solid protein in the presence of moisture. In addition to the possibility of affording stabilization by solid-phase dilution, such agents may be involved in interactions with water to promote stability or directly interact with proteins, preserving their structure upon lyophilization (Prestrelski *et al.* 1993a, 1993b). The former possibility was tested by examining the relationship between stabilizing potency and water sorbing capability, and the latter effect was investigated using recent advances in Fourier-transform infrared (FTIR) spectroscopy (Prestrelski *et al.* 1993a, 1993b; Singh *et al.*, 1993; Fu *et al.*, 1994; Griebenow and Klibanov, 1995) to determine secondary structural changes in various rHA formulations.

4.2 Lyophilization of rHA and Perturbations in Secondary Structure

rHA was initially obtained in an aqueous formulation. Because one goal was to test the stabilizing effects of various excipients, it was necessary to remove any additives already present in this solution prior to formulation into the lyophilized state. These consisted of sodium octanoate, which was removed by a standard defatting procedure (Chen, 1967), and salt, which was removed by dialysis. The succession of defatting, dialysis, and lyophilization resulted in a rHA solid containing negligible salts (~0.1 wt% of dry solid) and less than 0.1 mol-equivalent octanoate. In this case, the only other constituent present in the lyophilized rHA powder was water, at 10.1±1.7 g water/100g dry protein, averaged over all rHA preparations.

In order to investigate whether lyophilization resulted in any structural changes, we measured the IR spectrum in the amide III region of rHA in both aqueous solution (Figure 4.1A) and in the lyophilized form (Figure 4.1B). The data reveal marked

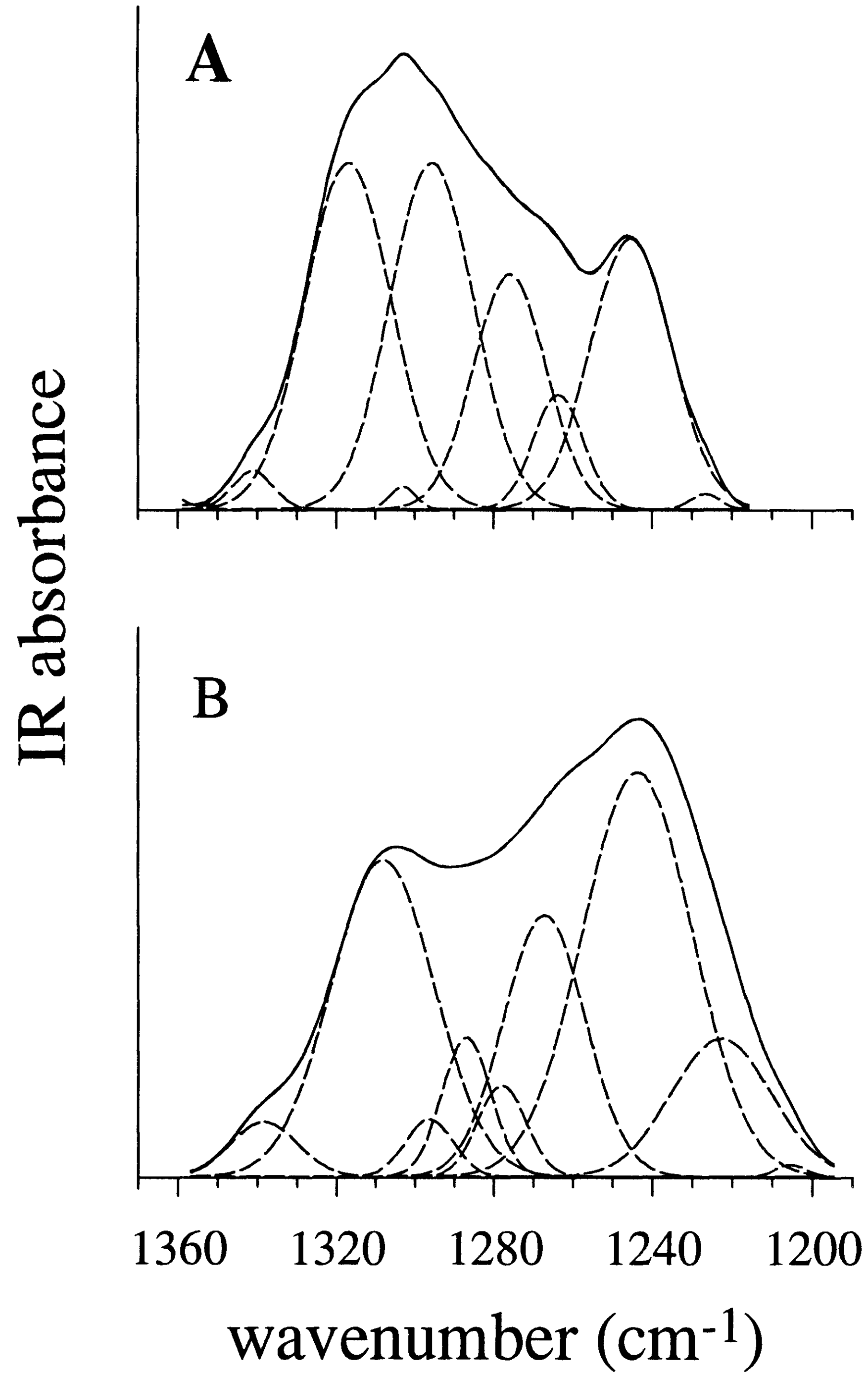


Figure 4.1 IR spectra of rHA in the amide III region and their Gaussian curve-fitting. (A) rHA dissolved in aqueous solution, pH 7.3; (B) rHA powder lyophilized from pH 7.3. The solid curves represent the superimposed original spectra and the Gaussian fit, and the dashed curves are individual Gaussian bands.

differences between the two. To quantify these differences, Gaussian deconvolution was performed on the spectra in order to calculate percentages of individual secondary structural elements. The curve-fitting results of the fit are presented in Table 4.1, with data for wavenumbers, relative areas, and structural assignments. Assignment of peak wavenumbers as determined by inspection of the second derivative of the spectra are in agreement with the results from the Gaussian curve-fit. These data are presented summarily in Table 4.2. (Table 4.2 also contains data for all subsequent Gaussian curve-fits for rHA, presented only as a summary of the data.)

In aqueous solution, the secondary structure of rHA consisted of 58% α-helix, with the remainder being unordered. (Note that this is in reasonable agreement with literature data, as indicated in Table 4.2.) Upon lyophilization, the α-helix content dropped to 30% with a concomitant rise in both β-sheet and unordered structural elements. This process was fully reversible; upon redissolution in water the secondary structural composition reverted to that in aqueous solution prior to lyophilization (Table 4.2). Thus, there was a net decrease in the structural order of rHA, reflecting partial unfolding. This phenomenon has implications for rHA's solid-state stability, as discussed below.

4.3 Kinetics of rHA Solid-Phase Aggregation

Having formulated rHA as a lyophilized powder, the next step was to choose the experimental conditions. The standard conditions chosen for this study (37°C and 96% relative humidity) resemble those within controlled-release polymeric delivery devices *in vivo* (Costantino *et al.*, 1994b). Under these conditions, lyophilized rHA absorbed 47±7 g water/100 g protein and exhibited a dramatic, time-dependent solubility loss, as revealed upon subsequent aqueous reconstitution (Figure 4.2A). The moisture was necessary to trigger the process since rHA that was not wetted (water content of 10.1±1.7 g water/g protein) did not exhibit any solubility loss. Size-exclusion chromatography (Figure 4.2B) showed that this solubility loss was due to aggregation. Initially (0 h), rHA was essentially monomeric (97%). After a 2- or 4-h incubation, considerable formation of

Table 4.1

Results of Gaussian curve-fitting of IR spectra in the amide III region for aqueous and lyophilized rHA[a]

Aqueous solution				**Lyophilized powder**[b]			
Peak position (cm^{-1})		area (%)	Assignment	Peak position (cm^{-1})		area (%)	Assignment
SD[c]	GCF[d]			SD	GCF		
1320±0	1316±1	30±1	α-helix	1312±0	1310±0	23±1	α-helix
1303±0	1302±1	1±0	α-helix	1302±1	1294±1	7±0	α-helix
1293±0	1295±1	27±4	α-helix	1287±1	1282±1	10±1	coil/turn
1281±1	1276±1	15±3	unordered	1277±1	1274±1	5±1	coil
1266±2	1264±0	4±1	unordered	1262±1	1263±0	11±1	coil
1244±1	1246±1	23±1	extended chain	1240±1	1247±1	29±1	extended chain
1227±1	1226±2	0	β-sheet	1221±0	1227±0	15±1	β-sheet

[a] Peak locations were initially identified by examination of the corrected (thrice smoothed with a 10-point function) second derivative of the original rHA spectra. Assignments of secondary structure was according to Griebenow and Klibanov (1995).

[b] The pH in aqueous solution prior to lyophilization was 7.3.

[c] SD:second derivative.

[d] GCF:Gaussian curve-fitting.

Table 4.2

Secondary structures of various rHA formulations as determined by Gaussian curve-fitting of IR spectra in the amide III region

Sample	α-Helix (%)	β-Sheet (%)	Unordered (%)
Aqueous solution, pH 7.3:			
no excipients[a]	58±4	0	42±3
in the presence of dextran[b]	54±3	1±1	45±3
Lyophilized (without excipients) from:			
pH 7.3[c]	30±1	15±1	55±1
pH 4.0	25±2	12±1	64±3
pH 9.0	35±1	10±1	55±1
Co-lyophilized with excipients[d]:			
NaCl, 1 g:6 g protein	32±1	17±1	52±1
dextran, 1 g:6 g protein	31±1	17±1	52±2
dextran, 1 g:2 g protein	38±1	13±2	49±4
dextran, 3 g:1 g protein	34±4	7±3	59±3
Spray dried	30±1	16±1	54±1
Rotary evaporated	25±2	17±1	58±3
Lyophilizate redissolved in water, pH 7.3:			
lyophilized without excipients[d]	55±2	1±1	44±1
containing dextran[b,d]	49±3	1±1	50±3

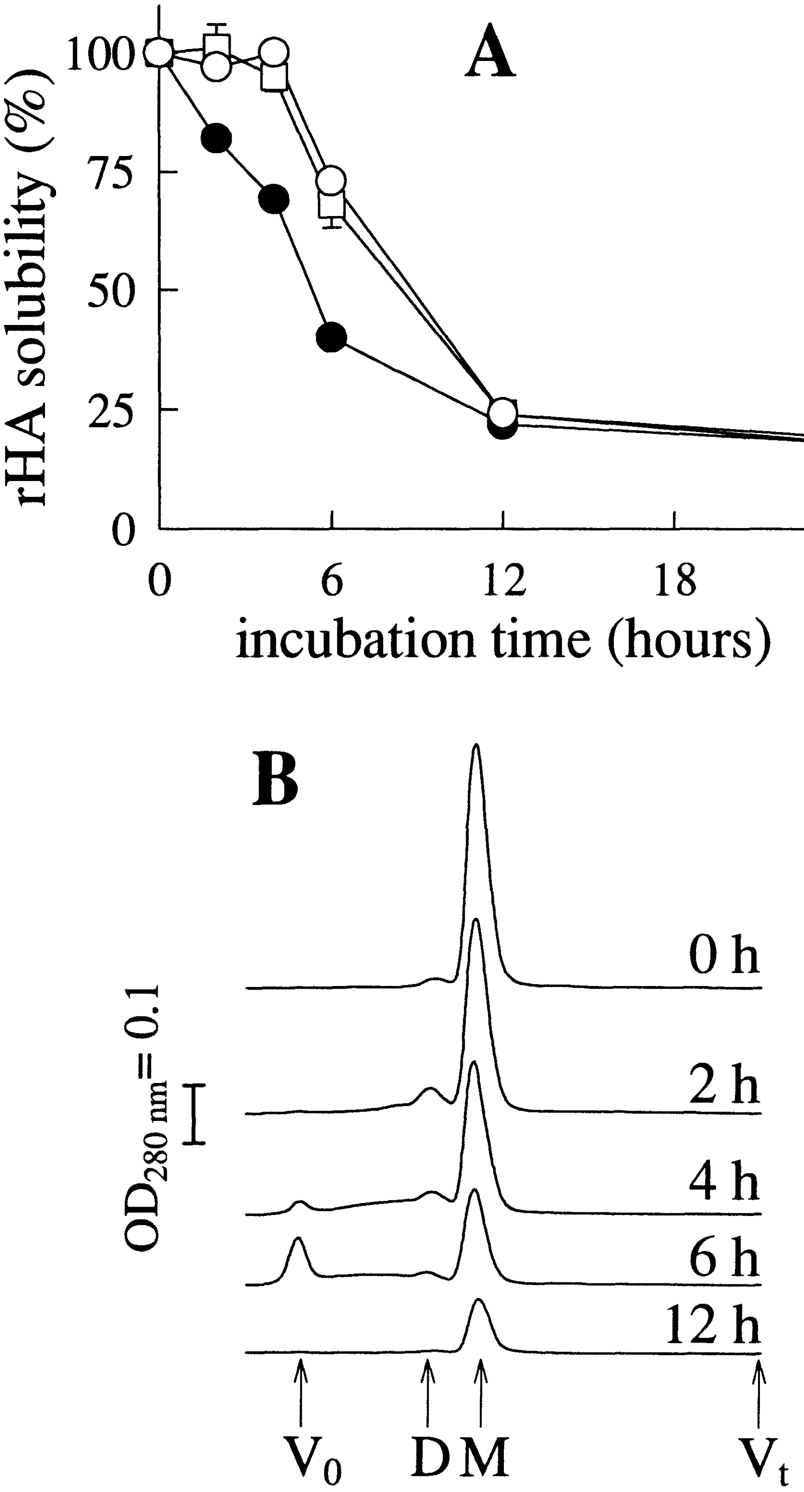
[a] Our calculated secondary structural composition is in reasonable agreement with the X-ray crystal structure (He and Carter, 1992) and CD data (Schechter and Blout, 1964).

[b] 3:1 (g/g) dextran (590 kDa)-to-rHA .

[c] Analysis of rHA lyophilized without excipients from 10 mg/ml yielded a secondary structure identical to that for protein lyophilized from 1 mg/ml.

[d] The pH in aqueous solution prior to lyophilization was 7.3.

Figure 4.2 Aggregation of lyophilized rHA at 37°C and 96% relative humidity. (A) Soluble rHA remaining as determined by [❑] total protein from the BCA assay, [○] total protein from size-exclusion chromatography (combined area of all protein peaks), and [●] rHA monomer from size-exclusion chromatography (area of the monomer peak). (B) Formation of moisture-induced rHA aggregates revealed by size-exclusion chromatography. Incubation time is denoted on each chromatogram. The following are indicated on the abscissa: V_0, void volume; D, rHA dimer; M, rHA monomer; V_t, total volume. Incubation conditions: 37°C and 96% relative humidity.


A
rHA solubility (%)
100
75
50
25
0
0
6
12
18
24
incubation time (hours)
B
$OD_{280\,nm} = 0.1$
0 h
2 h
4 h
6 h
12 h
V_0
D
M
V_t

soluble oligomers was observed, from dimers to large species eluting in the void volume. After a 6-h incubation, only approximately one third of soluble rHA was recovered. Evidently, rHA aggregates had grown such as to become water-insoluble. The remaining soluble species following longer incubations (≥12 h) were predominantly monomeric.

4.4 Mechanism of rHA Solid-Phase Aggregation

To determine the nature of protein-protein contacts resulting in the observed solubility loss, we attempted to re-dissolve the rHA aggregates. These data are depicted in Table 4.3. It was found that in the presence of a thiol reagent, 10 mM dithioerythritol (DTE) with 1 mM EDTA to prevent its autooxidation, complete solubilization was achieved, while no appreciable solubilization was observed without it even in 6 M urea or guanidine hydrochloride. This suggests that rHA aggregates were cross-linked through disulfide bonding rather than non-covalent interactions. The chemical mechanism is presumably the same as elucidated for moisture-induced aggregation of BSA (Liu *et al.*, 1991), namely intermolecular thiol-disulfide interchange (Fig. 2.6) The reactive groups in the process are the 17 disulfide bonds and the single free thiol (Cys^{34}), present in both bovine and human albumins (Peters, 1985). (For the case of BSA, only a partial solubilization of the aggregates was obtained in the presence of thiol reagent alone; some of the intermolecular disulfide cross-links could only be reduced when denaturant was also present.) However, not all of the Cys^{34} residues are in the form of reactive thiolate ions. Due to the formation of a mixed disulfide, either with plasma cysteine or glutathione, and possible further loss during processing and/or storage, commercially available albumin usually contains 0.5-0.7 mol thiol/mol albumin (Peters, 1985). For example, the BSA used in the moisture-induced aggregation study had a thiol content of 0.66 mol equivalents (Liu *et al.*, 1991). The rHA employed in the present investigation had 0.78-0.88 mol-equivalents thiol following its defatting, dialysis and lyophilization. (This represents a slight loss from 1.06±0.06 mol-equivalents found in the initial aqueous formulation provided by the supplier.) Considering these similarities, it is not surprising that the mechanisms of moisture-induced aggregation are the same for lyophilized BSA and rHA.

Table 4.3

Solubility of moisture-induced rHA aggregates in various solutions[a]

Solvent	rHA aggregates solubility (%)[b]
PBS[c]	0[d]
PBS + 6 M urea	0
PBS + 6 M urea + 1 mM EDTA	3
PBS + 6 M urea + 10 mM DTE[e] + 1 mM EDTA	93
PBS + 6 M guanidine hydrochloride	0
PBS + 6 M guanidine hydrochloride + 10 mM DTE + 1 mM EDTA	101
PBS + 10 mM DTE + 1 mM EDTA	97

[a] Samples of lyophilized rHA (2 mg) were incubated at 37°C and 96% relative humidity for one day, and then reconstituted in various aqueous solutions (2 ml) by stirring for 30 min. The insoluble fraction was then removed by filtration (Millex-GV, Millipore) and the soluble fraction assayed for total protein using unincubated rHA in the same solution as the standard.

[b] Note that separate calibration curves were obtained for protein determinations in the presence of the additives listed.

[c] PBS:phosphate-buffered saline.

[d] Denotes that the amount of aggregates dissolved in these solvents was ≤ 0 (amount of unincubated rHA dissolved).

[e] DTE:dithioerythritol.

4.5 Inhibition of rHA Aggregation by Co-Lyophilization with Excipients

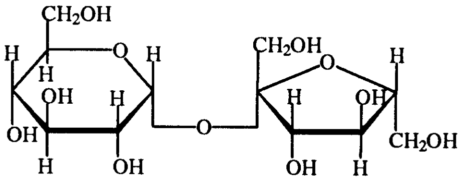
One approach for stabilizing rHA against moisture-induced aggregation is by co-lyophilizing the protein with excipients. These excipients can stabilize rHA by various scenarios including: (1) they can reduce intermolecular contacts between protein molecules; (2) they can preserve protein structure via excipient-protein interactions, and; (3) they can maintain the water activity at levels which are not conducive to aggregation. Herein, we will explore the stabilizing effect of various excipients and determine which of these modes predominate.

4.5.1 Effect of co-lyophilization with several classical excipients. It was reasonable to expect that one of the means reported for stabilizing BSA towards moisture-induced aggregation, namely dilution achieved by co-lyophilization with water-soluble polymeric excipients (Liu *et al.*, 1991), would also be beneficial for rHA. To test this, we determined stability of rHA co-lyophilized (50 wt% on a dry basis, or 1 g:1 g dry rHA) with two polymeric excipients, dextran and poly[ethyleneglycol] (PEG). Three low-molecular-weight excipients, sorbitol, sucrose and trehalose (also co-lyophilized at 1 g:1 g dry rHA) were also tested for their ability to stabilize rHA. (A weight basis, rather than a mole basis, was chosen so that data for low- and high-molecular weight compounds may be compared in a meaningful manner.) These excipients (structures shown in Figure 4.3A) were logical initial models for study since they are commonly employed to stabilize proteins in solution and during lyophilization (Arakawa *et al.*, 1991; Timasheff, 1992).

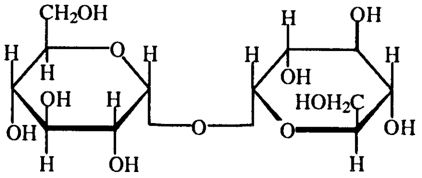
The solubilities of these excipient:rHA lyophilizates following incubation at the standard conditions are shown in Figure 4.4. All of the above mentioned compounds, with the notable exception of PEG, were completely successful in stabilizing rHA towards the formation of insoluble aggregates even after the longest incubation period of four days. It is not clear why PEG was beneficial for stabilizing BSA as previously reported (Liu *et al.*, 1991) and not rHA herein; however, it should be noted that whereas lyophilized BSA was directly mixed with water and then incubated, in the present investigation we have wetted the protein via sorption directly from the vapor phase.

Figure 4.3 Structures of various excipients co-lyophilized with rHA. (A) Classical lyoprotectants. (B) Dextran derivatives. (C) Sorbitol-related compounds. (D) Other compounds.

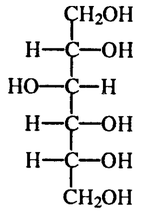
A



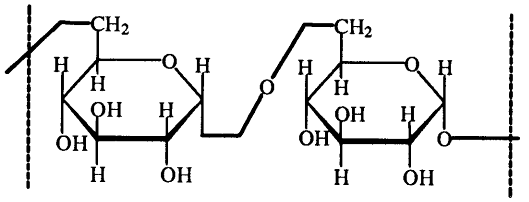
sucrose



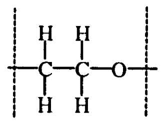
trehalose



D-sorbitol

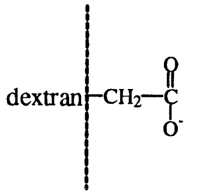


dextran

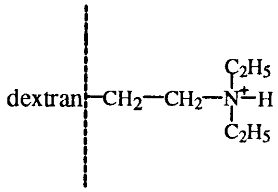


PEG

B

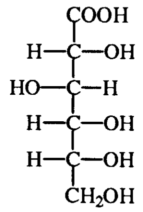


CM-dextran

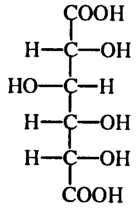


DEAE-dextran

C



D-gluconic acid



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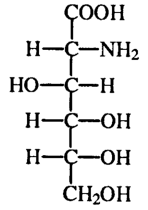
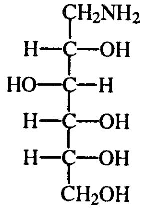
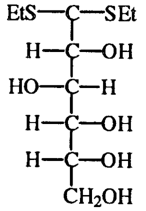


D-glucosaminic acid

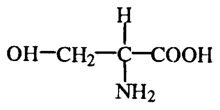


D-glucamine



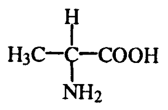
D-glucose
diethylmercaptal

D



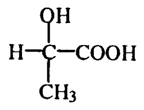
$OH{-}CH_2{-}\overset{H}{\underset{NH_2}{C}}{-}COOH$

L-serine



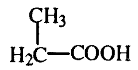
$H_3C{-}\overset{H}{\underset{NH_2}{C}}{-}COOH$

L-alanine



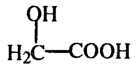
$H{-}\overset{OH}{\underset{CH_3}{C}}{-}COOH$

L,D-lactic acid



$\overset{CH_3}{H_2C}{-}COOH$

propionic acid



$\overset{OH}{H_2C}{-}COOH$

glycolic acid

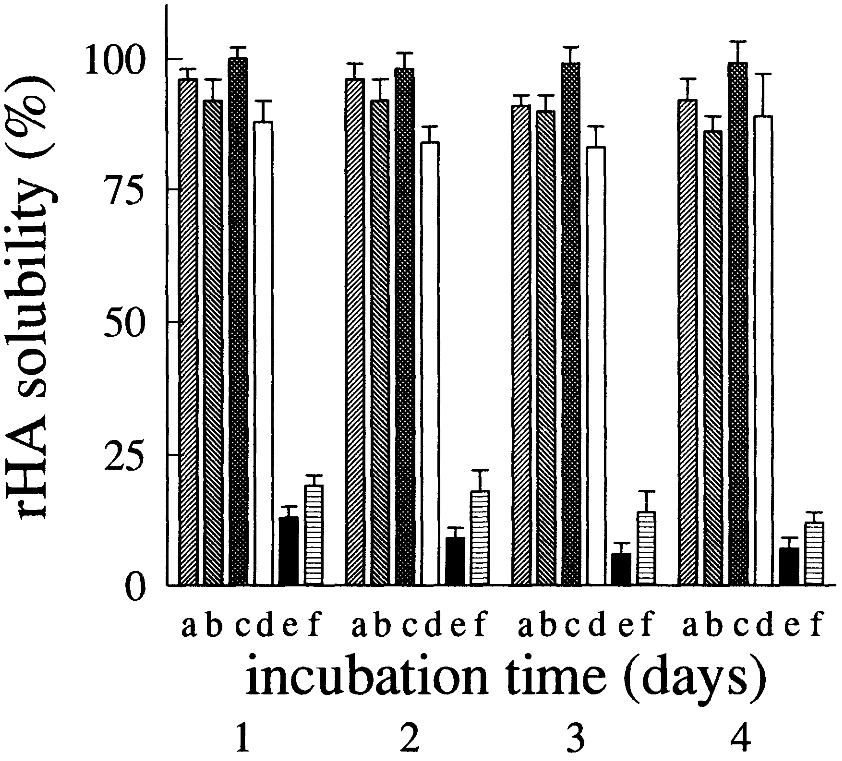


Figure 4.4 Stability of rHA co-lyophilized with various classical excipients. Data were obtained for rHA co-lyophilized (50 wt%, dry basis) with: a, sorbitol; b, sucrose; c, trehalose; d, dextran 590 kDa MW; e, poly[ethylene glycol] 20 kDa MW; f, without excipients. Incubation conditions are the same as in Fig. 4.2.

4.5.2 Effect of co-lyophilization with varying amounts of dextran. If excipients inhibit solid-phase aggregation by dilution, then the extent of stabilization should depend upon the amount of additive present. To explore this, dextran was co-lyophilized with rHA in varying amounts, and these samples were tested for moisture-induced aggregation. As seen in Figure 4.5A, the stability of rHA greatly improved as the dextran-to-rHA ratio was increased from 1:6 to 1:1 (g/g). When the level of co-lyophilized dextran was 1:1 or greater, rHA was completely stable against the moisture-induced aggregation; no solubility loss was observed even after a 4-day incubation. The relationship between the amount of dextran present and the stability it imparted can also be seen in Figure 4.5B, where the solubility loss is plotted for the various formulations at a fixed incubation period of one day.

To verify that the stabilization was not due to variation in water content during the high-humidity incubation, the water uptakes of various dextran:rHA co-lyophilizates were measured and compared with those for the excipient and the protein lyophilized individually. The water uptake during the high-humidity incubation was independent of the dextran-to-rHA ratio in the lyophilized powder (Table 4.4). Because the stability of rHA varied widely in this range (Fig. 4.5; Table 4.4), the mechanism of stabilization imparted by dextran cannot be the result of any excipient-water interactions.

Since sugars and polyols are known to enhance protein stability upon dehydration (Arakawa *et al.*, 1991, Prestrelski *et al.* 1993a, 1993b), we investigated the secondary structure of rHA co-lyophilized with dextran at three extreme dextran-to-rHA (g/g) ratios: 1:6, at which rHA was only slightly stabilized; 1:2, at which rHA was significantly stabilized; and 3:1, at which rHA was completely stabilized (Fig. 4.5). The FTIR data reveal only minor differences in the secondary structural composition of the lyophilized dextran:rHA formulations compared to that of rHA lyophilized alone (Table 4.2). The pronounced common trend observed is a significant loss of α-helical structure and an increase in β-sheet and unordered elements upon lyophilization. Increasing the fraction of co-lyophilized dextran resulted in slightly lower β-sheet formation upon dehydration. For example, at the dextran-to-rHA ratio of 1:6, the secondary structural composition was 31% α-helix, 17% β-sheet, and 52% unordered (which is very similar to the composition

Figure 4.5 Stabilization of rHA afforded by co-lyophilized dextran. (A) Time course of solubility of rHA co-lyophilized with dextran at various dextran-to-rHA weight ratios, as indicated. The dashed line depicts the time course of rHA aggregation in the absence of dextran (from Fig. 4.2). (B) rHA solubility after a one-day incubation as a function of dextran-to-rHA weight ratio. Incubation conditions are the same as in Fig. 4.2

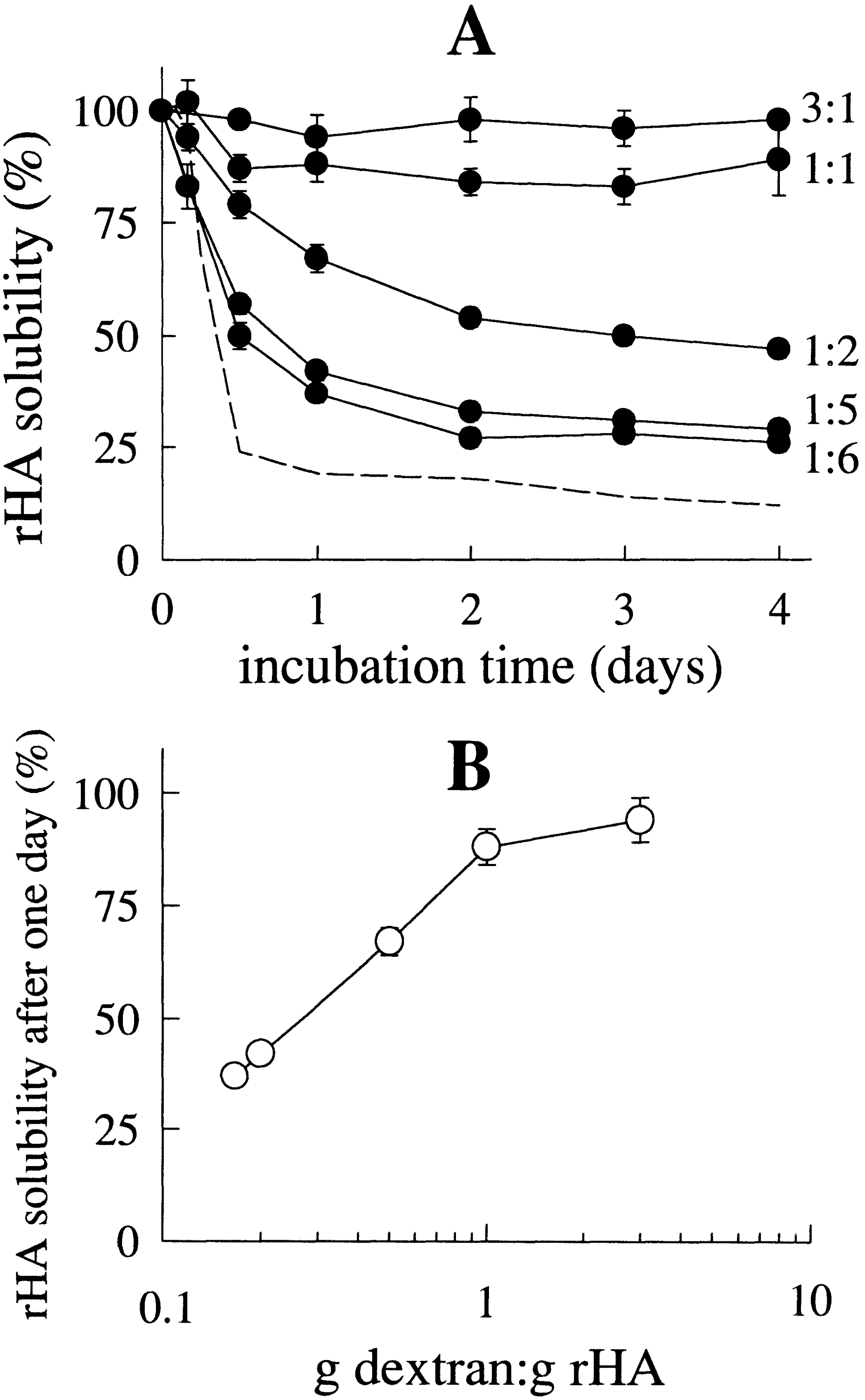

A
rHA solubility (%)
100
75
50
25
0
0
1
2
3
4
incubation time (days)
3:1
1:1
1:2
1:5
1:6
B
rHA solubility after one day (%)
100
75
50
25
0
0.1
1
10
g dextran:g rHA

Table 4.4

Aggregation and water sorption of rHA co-lyophilized with varying amounts of dextran

Formulation (g dextran:g rHA)	Aggregation[a] (%)	Water content, wetted[a] (g water/100 g dry solid)
0 (rHA alone)	81±2	47±7
1:6	63±2	nd[b]
1:5	58±2	48±5
1:2	33±3	41±7
1:1	12±4	40±2
3:1	6±5	54±7
dextran	na[c]	47±7

[a] Aggregation (solubility loss) and water contents were determined following a one-day incubation at 37°C and 96% relative humidity.

[b] Not determined.

[c] Not applicable.

of rHA lyophilized alone), and at 3:1 the secondary structural composition was 34% α-helix, 7% β-sheet, and 59% unordered. Further examination of the latter sample demonstrated that dextran did not alter the secondary structural composition of rHA in aqueous solution, and that the structural reordering upon lyophilization was nearly completely reversible (Table 4.2).

Since the protection of rHA by dextran cannot be explained by either preservation of the protein structure upon lyophilization or increased water sorption during incubation in the humid environment, the most likely rationale is that dextran acts as a 'dilutant' in the solid phase, decreasing protein-protein contacts and hence slowing down intermolecular processes (Liu *et al.*, 1991) This view is consistent with our FTIR findings. Note that prevention of protein-protein interactions is likely to lower the β-sheet content of the lyophilized powders (Griebenow and Klibanov, 1995), as indeed observed (Table 4.2).

4.5.3 Effect of co-lyophilization with charged dextrans. Charged derivatives of dextran were also tested for their ability to stabilize rHA. These were carboxymethyl (CM)-dextran and diethylaminoethyl (DEAE)-dextran (structures shown in Fig. 4.3B). The amount added was 1 g:6 g dry rHA, where uncharged dextran had only a slight stabilizing effect. Both CM- and DEAE-dextrans had greater stabilizing action compared to their uncharged form (Figure 4.6). Apparently, the presence of a charge increases an excipient's ability to stabilize rHA during incubation at standard conditions. This result suggests that there may be another mode of stabilization, of which two have already been mentioned: excipient-protein interactions and excipient-water interactions.

4.5.4 Effect of co-lyophilization with varying amounts of sorbitol. Since both low- and high-molecular weight excipients may be successful in stabilizing rHA against aggregation (Fig. 4.4), it was logical to choose sorbitol and other low-molecular-weight compounds (rather than dextran or other polymeric ones) for further investigation of potential excipient-water or excipient-protein interactions. This latter scenario is of note considering that sorbitol has been implicated as a preserver of protein structure upon lyophilization, as reported in a hydrogen isotope exchange/NMR study of bovine

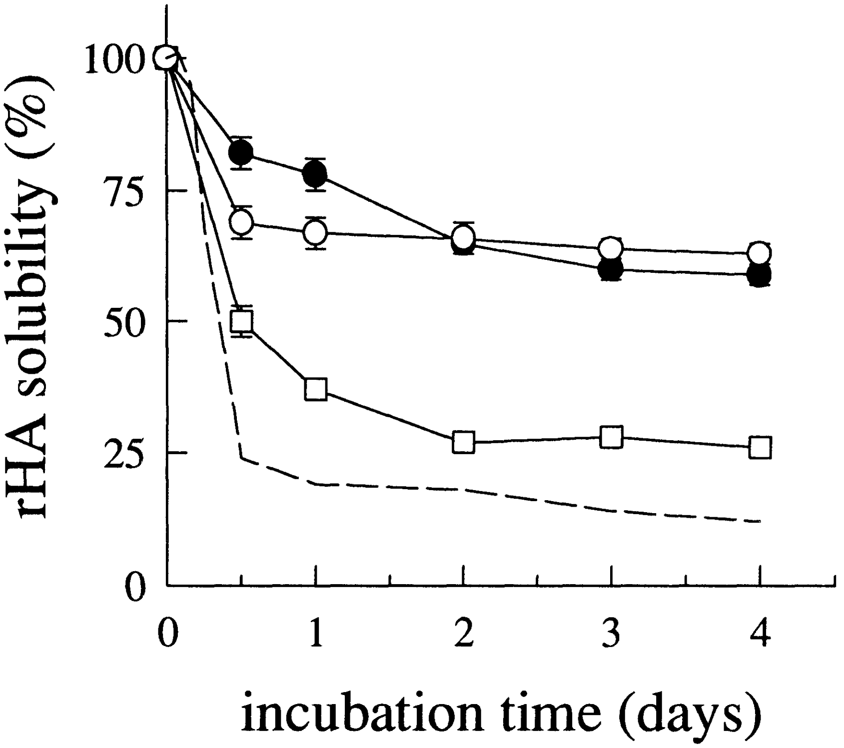


Figure 4.6 Stabilization of rHA afforded by charged derivatives of dextran. Data were obtained for rHA co-lyophilized with 1 g:6 g dry protein of: [○] DEAE-dextran; [●] CM-dextran; and [☐] dextran. The dashed line depicts the time course of rHA aggregation in the absence of dextran (from Fig. 4.2). Incubation conditions are the same as in Fig. 4.2.

pancreatic trypsin inhibitor (Desai *et al.*, 1994). This effect is important because conformational stability is often linked with aggregation and other protein degradation pathways (Manning *et al.*, 1989; Cleland *et al.*, 1993). If sorbitol and protein interact promoting physical stability, then it is conceivable that this would contribute to the inhibition of aggregation in the sorbitol:rHA lyophilizates.

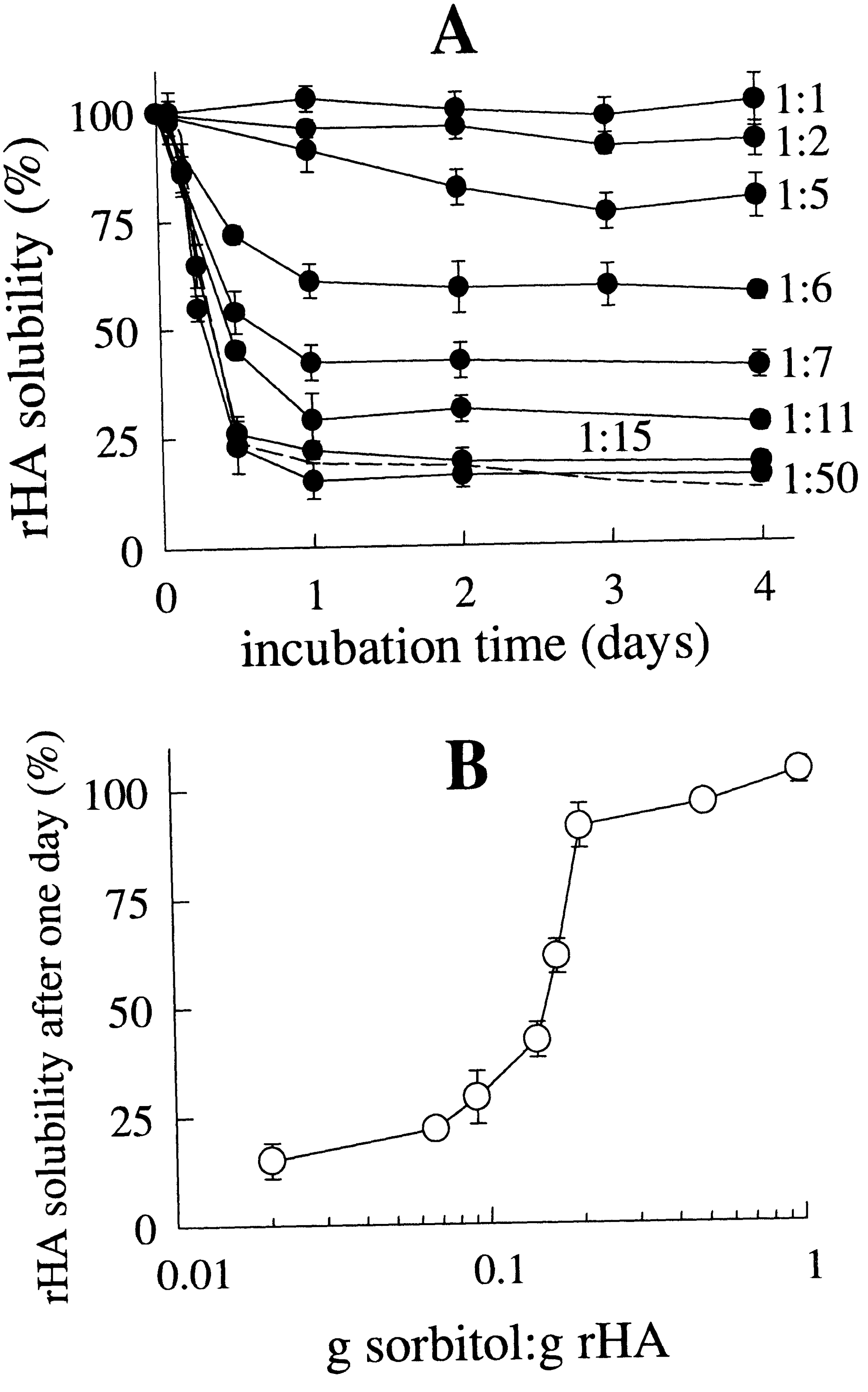
To test this, we followed the solubility loss of rHA co-lyophilized with various levels of sorbitol (Figure 4.7A). As was the case for dextran (Fig 4.5A), increased stability was achieved at higher excipient loadings. At the highest sorbitol-to-rHA ratios tested, 1:1 and 1:2 (g:g dry rHA), essentially complete stabilization was achieved (Fig. 4.7A). Aggregation of rHA with a sorbitol:rHA ratio of 1:5 was appreciable but slight – a 21±5% solubility loss after a four-day incubation. As the sorbitol content was lowered further to 1:11, the solubility loss, and hence the extent of aggregation, increased dramatically. At still lower sorbitol contents, 1:15 and 1:50, aggregation was similar to that of rHA lyophilized without sorbitol.

For rHA formulated over the relatively narrow range of sorbitol content from 1:5 to 1:7, there were markedly different stabilities. This effect can be more clearly seen in Figure 4.7B, where the rHA solubility following a one-day incubation has been plotted against the sorbitol-to-rHA weight ratio. Note here that the sigmoidal dependence observed for sorbitol is different from that observed for dextran (Fig 4.5B) suggesting that another stabilization mechanism, besides dilution, may be involved.

4.5.5 Effect of co-lyophilization with various low-molecular-weight excipients.

In order to investigate the relationship between an excipient's molecular structure and its stabilizing power, we tested a variety of compounds similar in structure to sorbitol for their ability to inhibit moisture-induced aggregation. The structures of these compounds are shown in Fig. 4.3C. The amount of each compound added was 1 g:6 g dry rHA; this level of sorbitol only partially stabilized rHA (Fig. 4.7), thus allowing the detection of both greater and lesser stabilization. The data obtained (Figure 4.8) indicate that the presence of a charged group appears to enhance an excipient's ability to protect rHA. Compounds with either a carboxyl group (D-gluconic acid; curve b in Fig. 4.8) or an amino group (D-

Figure 4.7 Stabilization of rHA afforded by co-lyophilized sorbitol. (A) Time course of solubility of rHA co-lyophilized with sorbitol at various sorbitol-to-rHA weight ratios, as indicated. The dashed line depicts the time course of rHA aggregation in the absence of sorbitol (from Fig. 4.2). (B) rHA solubility after a one-day incubation as a function of sorbitol-to-rHA weight ratio. Incubation conditions are the same as in Fig. 4.2.


A
rHA solubility (%)
100
75
50
25
0
0
1
2
3
4
incubation time (days)
1:1
1:2
1:5
1:6
1:7
1:11
1:15
1:50
B
rHA solubility after one day (%)
100
75
50
25
0
0.01
0.1
1
g sorbitol:g rHA

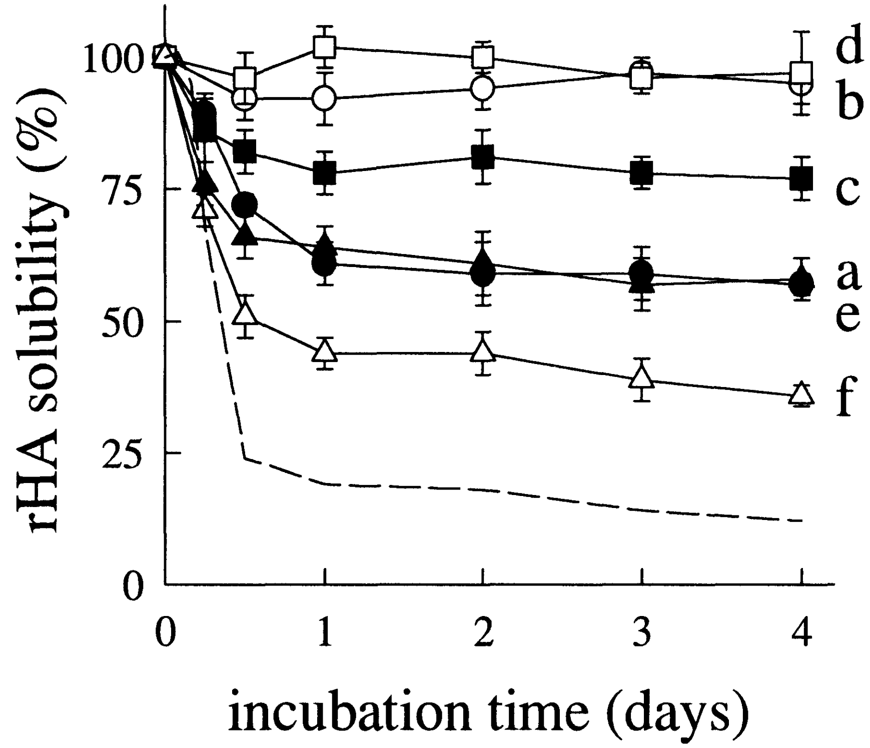


Figure 4.8 Stabilization of rHA afforded by addition of 1 g:6 g rHA of sorbitol and similar compounds. The added compounds were as follows: a, sorbitol; b, D-gluconic acid; c, D-glucaric acid; d, D-glucamine; e, D-glucosaminic acid; f, D-glucose diethyl mercaptal. The dashed line depicts the time course of rHA aggregation in the absence of excipient (from Fig. 4.2). Incubation conditions are the same as in Fig. 4.2.

glucamine; curve d) present at C-1 were completely successful at stabilizing rHA against aggregation. However, D-glucosaminic acid (curve e), which contains both a carboxyl group at C-1 and an amino group at C-2, provided only the same stabilization as sorbitol (curve a). D-Glucaric acid (curve c), which contains two carboxyl groups (at C-1 and C-6), stabilized rHA to a greater extent than sorbitol, but not as effectively as D-gluconic acid. Finally, D-glucose diethyl mercaptal (curve f) was somewhat less potent than sorbitol, possibly because it contains fewer hydroxyl groups per weight unit than the other compounds depicted in Fig. 4.8.

To explain these confusing effects, we decided to investigate more simple compounds still containing the same structural elements shown to influence the extent of stabilization (hydroxyl, amino, and carboxyl groups) but having fewer carbon atoms. These consisted of two amino acids, serine and alanine, two hydroxy acids, lactic and glycolic, as well as an unfunctionalized carboxylic acid, propionic (structures shown in Fig. 4.3D). Stability data for these compounds are presented in Figure 4.9. Serine and alanine (curves a and b, respectively, in Fig. 4.9) both afforded moderate protection of rHA: the former imparted about the same stabilization as sorbitol (curve a on Fig. 4.8), whereas the latter did not perform as well. All the other acids afforded essentially complete stabilization.

These data (Figs. 4.8 and 4.9) appeared puzzling given the excipient-protein interaction usually implicated in increasing the stability of lyophilized proteins. It has been hypothesized that hydroxyl groups present in sugars and other polyols hydrogen bond (acting as both H acceptors and donors) with proteins, thus serving as "water substitutes" to insure conformational stability (Arakawa *et al.*, 1991; Prestrelski *et al.*, 1993a, 1993b). (This effect would be important for albumin because its conformation would affect aggregation via thiol-disulfide interchange as discussed below.) However, this is not the predominant stabilization mechanism responsible in the case of the low-molecular-weight excipients. For example, this mechanism cannot explain why glucaric acid was more potent than sorbitol but less potent than gluconic acid in stabilizing rHA (Fig. 4.8). Furthermore, it would be impossible to explain why a compound as simple as sodium chloride was as effective (curve g in Fig. 4.9) as the most potent stabilizers.

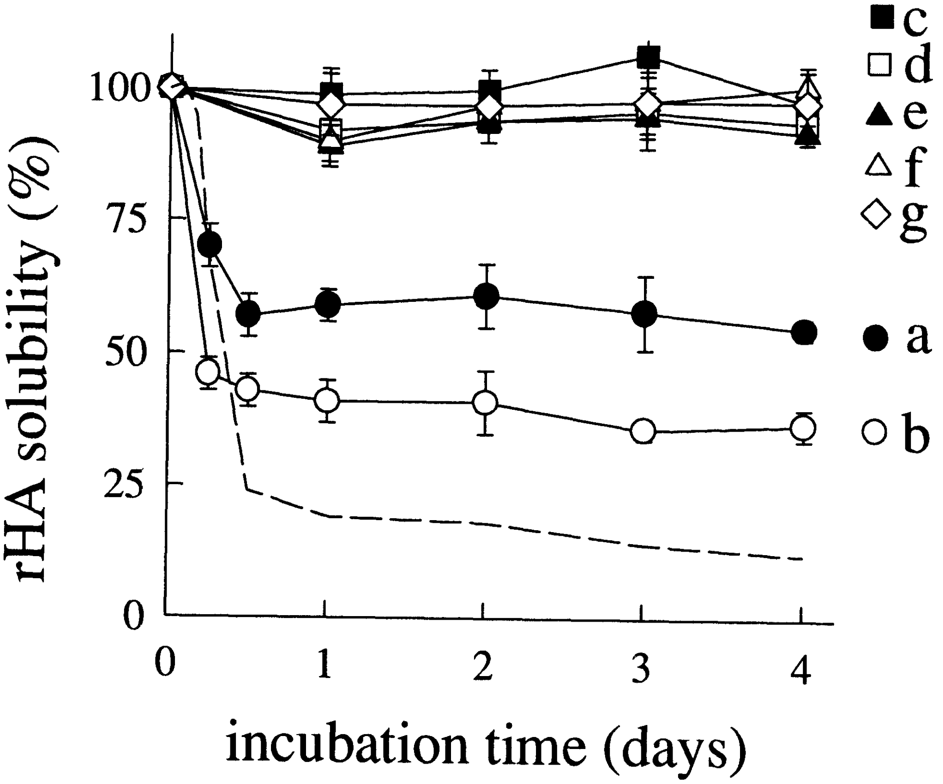


Figure 4.9 Stabilization of rHA afforded by addition of 1 g:6 g rHA of various compounds. The compounds added were as follows: a, L-serine; b, L-alanine; c, L-lactic acid; d, D-lactic acid; e, glycolic acid; f, propionic acid; g, sodium chloride. The dashed line depicts the time course of rHA aggregation in the absence of excipient (from Fig. 4.2). Incubation conditions are the same as in Fig. 4.2.

4.6 Effect of Moisture on rHA Solid-Phase Aggregation

One cannot formulate a mechanistic hypothesis explaining the stabilization afforded by excipients co-lyophilized with rHA on the basis of their direct interactions with the protein. In addition, simple dilution of protein molecules by co-lyophilized excipients (Liu *et al.*, 1991) cannot be responsible for the marked differences observed in the degree of stabilization imparted by various low-molecular-weight compounds. Consequently, we examined the effect of excipients on the only other component of the system under investigation, namely water.

4.6.1 Effect of water content on rHA stability in the presence of co-lyophilized excipients. It is becoming increasingly clear that solid protein aggregation and water content are critically linked (Hageman, 1988; Hageman, 1992). To explore this relationship, we lyophilized each excipient (in the absence of rHA) and measured water sorption by them following a one-day incubation at standard conditions. The data obtained were then compared with each compound's ability to retard rHA aggregation (under identical conditions) when present at 1 g:6 g dry rHA (data taken from Figs. 4.8 and 4.9). In this analysis, we also included the data for additional compounds in order to expand the scope of our correlation. These compounds were the disaccharides sucrose and trehalose, the polysaccharide dextran as well as its carboxymethyl and diethylaminoethyl derivatives, and a structurally unrelated polymer, PEG.

The results (Figure 4.10) indicate that a compound's effectiveness in stabilizing rHA roughly correlates with its water uptake: the greater the excipient's affinity for water, the greater, in general, is its stabilizing potency. This observation, based on a wide range of diverse compounds, indicates that the stabilization primarily arises not from direct interactions of excipient with rHA, but rather from interactions between excipient and water.

Because water sorption of mixtures can be estimated from the data for individual components (Lang and Steinberg, 1980; Bakhit and Schmidt, 1992, 1993), we conclude that rHA stability rises with increasing amount of water sorbed by the excipient:rHA

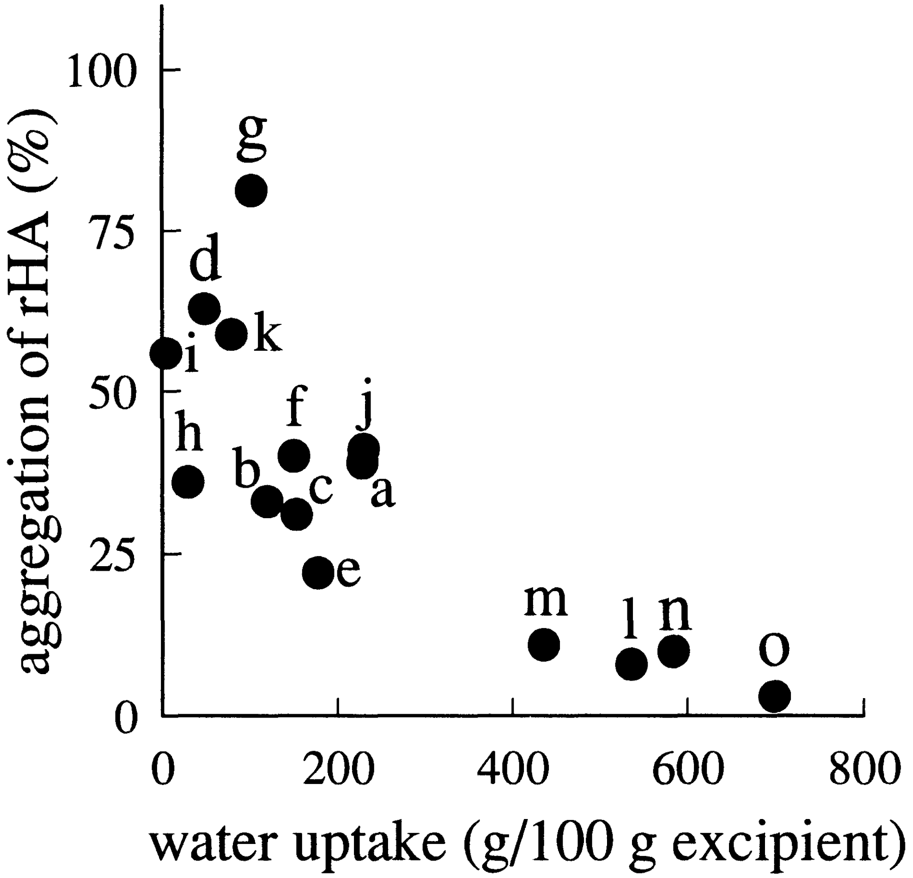


Figure 4.10 Correlation between an excipient's ability to stabilize rHA and its water uptake. The abscissa represents the amount of water sorbed by pure excipient (lyophilized from 1 mg/ml, pH 7.3), and the ordinate depicts the aggregation of rHA (solubility loss) in a 1 g excipient:6 g rHA lyophilizate. The excipients were as follows: a, sorbitol; b, sucrose; c, trehalose; d, dextran; e, carboxymethyl-dextran; f, diethylaminoethyl-dextran; g, poly(ethylene glycol); h, D-glucosaminic acid; i, D-glucose diethyl mercaptal; j, L-serine; k, L-alanine; l, D-lactic acid; m, glycolic acid; n, propionic acid; o, sodium chloride. (D-Gluconic acid, D-glucaric acid, and D-glucamine were omitted from this analysis since they could not be lyophilized to yield a homogeneous powder.) Incubation conditions are the same as in Fig. 4.2.

system. Under our conditions (i.e., 96% relative humidity) many of the compounds tested exhibit multilayer water sorption (Sloan and Labuza, 1975a, 1975b). Water sorbed in multilayers has a mobility similar to that of bulk water and thus should be available to the neighboring protein molecules. In this manner, an excipient can act as a "sponge", drawing moisture from the humidified atmosphere and bringing it in close contact with the protein.

4.6.2 Effect of water content on rHA stability without excipients. To verify that rHA stability is indeed influenced by the level of hydration, we monitored aggregation as a function of water content of the lyophilized protein (no excipient). The data (Figure 4.11), obtained after a one-day incubation at 37°C, exhibit a pronounced bell-shaped dependency with maximum aggregation corresponding to 47±7 g water/100 g dry protein, i.e., to 96% relative humidity (standard conditions). During storage at this humidity, the presence of a strongly water-sorbing excipient would increase rHA's hydration, thus pushing its behavior along the descending portion of the curve in Fig. 4.11. This rationale is consistent with our observation that during incubation at 96% relative humidity, excipients with greater water-sorbing capacities were, in general, more potent at stabilizing rHA (Fig 4.10). (Note that according to this mechanism, upon incubation at lower relative humidities, there may be either no effect or even destabilization by excipients, depending on the final moisture level in the vicinity of the rHA.)

4.6.3 Rationalization of the dependence of rHA aggregation on moisture content and the refolding hypothesis. The bell-shaped dependency depicted in Fig. 4.11 warrants further discussion. A very similar result was obtained for BSA (Fig. 2.10) where the maximum aggregation was observed at water contents between about 35-55 g water/100 g dry protein. In that report (Liu *et al.*, 1991), the ascending portion of the curve was attributed to rising conformational mobility of the protein molecule upon increasing hydration, which in turn should promote the intermolecular thiol-disulfide reaction. The descending portion of the curve was attributed to dilution of reactants upon hydration, or an increase in the distance between protein molecules. This is also a

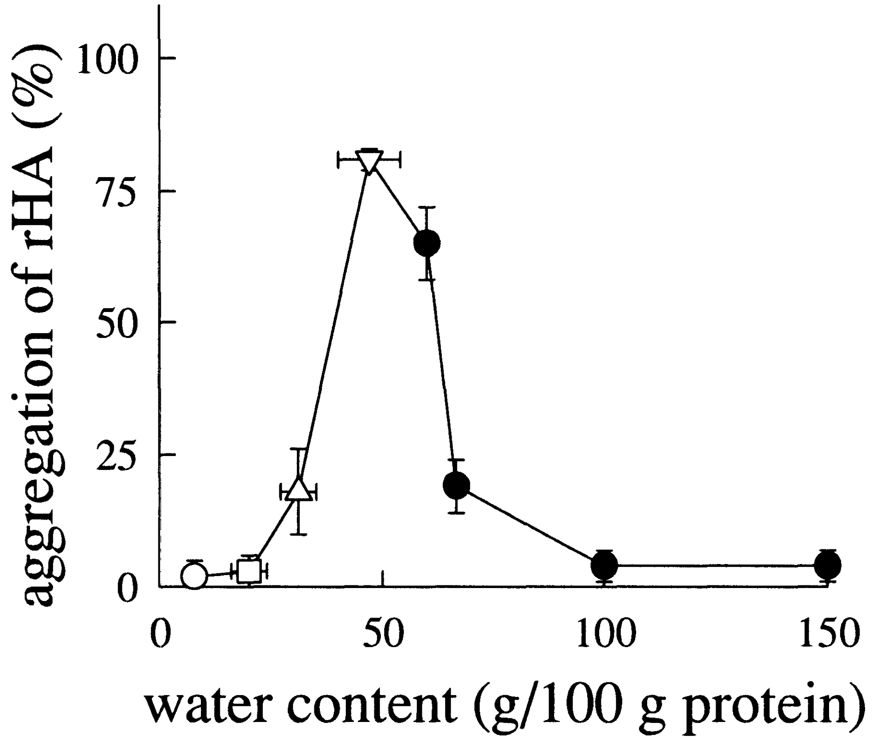


Figure 4.11 Dependence of rHA solid-phase aggregation on moisture content. Water content was measured following incubation at various relative humidities: [○], 11%; [□], 75%; [Δ], 90%; [∇], 96%. Higher water contents [●] were achieved by directly adding water (pH 7.3) to the lyophilized rHA. Incubation conditions: one day at 37°C.

potential explanation for rHA's increased stability in the presence of water-sorbing excipients. These excipients draw water into the system, and this water (not the excipient itself) dilutes the protein, thus enhancing its stability. However, this latter scenario is unlikely herein considering the water sorption data for the various sorbitol:rHA lyophilizates (Table 4.5). Over the range of sorbitol contents in which a dramatic increase in stability was observed (1:7 to 1:5 g:g dry protein), water uptake was not statistically different. Dilution by water (or even by both water and sorbitol) cannot explain the enhanced stability over such a narrow range in sorbitol and water contents.

The most likely explanation for the descending portion of the curve involves water's critical role in maintaining protein structure. Because water is directly involved in such forces as hydrogen bonding, electrostatic interactions, and the hydrophobic effect, that are responsible for maintaining the folded conformation of proteins (Creighton, 1983; Dill, 1990), its removal (e.g., by lyophilization) should affect the balance of these forces (Kuntz and Kauzman, 1974) and thus alter protein conformation (Prestrelski *et al.*, 1993a, 1993b; Desai *et al.*, 1994; Griebenow and Klibanov, 1995). (In many cases, this denaturation is reversible since native structure is achieved upon aqueous reconstitution (Prestrelski, 1993a; Desai *et al.*, 1994).) Indeed, as discussed earlier (Section 4.2), there was a significant alteration in rHA's secondary structure upon lyophilization, as revealed by FTIR spectroscopy in the amide III region (spectra shown in Fig 4.1; assignment of secondary structure via Gaussian deconvolution listed in Table 4.2). Specifically, there was a partial unfolding of the rHA molecule, as evidenced by a transition from α-helical elements to β-sheet and unordered structures.

There are several reasons to expect rHA conformation to be critically linked to aggregation via thiol-disulfide interchange. First, aggregation of albumin via thiol disulfide interchange does not occur in aqueous solution under conditions that favor the folded conformation. For example, even when stirred for several days in aqueous solution at 37°C and pH 7.3, albumin does not aggregate (Liu *et al.*, 1991). This is most likely due to the fact that under these conditions, although the Cys^{34} residue is exposed to the solvent (and hence eligible for reaction) the disulfides are almost entirely inaccessible (Diez *et al.*, 1964; Habeeb 1966b; Habeeb, 1978; He and Carter, 1992).

Table 4.5

Aggregation and water sorption of rHA co-lyophilized with varying amounts of sorbitol

Formulation (g sorbitol:g rHA, dry basis)	Aggregation (%)[a]	Water content, wetted[a] (g water/ 100g dry solid)
0 (rHA, no added sorbitol)	81 ± 2	47 ± 7
1:50	85 ± 4	nd[b]
1:15	78 ± 2	52 ± 5
1:11	71 ± 6	56 ± 3
1:7	58 ± 4	56 ± 11
1:6	39 ± 4	59 ± 5
1:5	9 ± 5	57 ± 4
1:2	4 ± 2	103 ± 17
1:1	0 ± 3	143 ± 19
sorbitol (no added rHA)	na[c]	228 ± 26

[a] Aggregation (solubility loss) and water contents were determined following a one-day incubation at 37°C and 96% relative humidity.

[b] Not determined.

[c] Not applicable.

Second, it has been reported that thiol-disulfide interchange does occur to albumin in aqueous solution under conditions that favor unfolding, or significant perturbations of albumin's three-dimensional structure. Examples of such conditions are alkaline pH and temperatures above 65°C (Aoki *et al.*, 1973), and alkaline pH and the presence of high levels of denaturing agents urea or guanidine hydrochloride (Andersson, 1969). These reports are consistent with the observation that extreme of pH and presence of denaturing agents dramatically increases the accessibility of the disulfide bonds of albumin, thus increasing the likelihood of thiol-disulfide interchange (Habeeb, 1978). In fact, in aqueous solution, the formation of human albumin aggregates via disulfide bonds occurring at ≥75°C directly correlated with unfolding of the protein molecule (Wetzel *et al.*, 1980).

Third, when the physical stability of albumin is increased by the binding of fatty acids, enhanced stability towards thiol-disulfide interchange is observed (Sogami *et al.*, 1969; Brandt and Andersson, 1976). The binding of fatty acids to albumin results in a more compact structure (Soetewey *et al.*, 1972; Dzhafarov, 1992). Furthermore, when fatty acids are bound the accessibility of disulfides may significantly decrease, in particular at denaturing conditions which ordinarily result in very high disulfide accessibility (Habeeb, 1978). We have found that when rHA was lyophilized following dialysis (but not defatting), 2.4±0.6 mol octanoate/mol rHA were bound and this protein exhibited only a 30±3% solubility loss as compared to the 81±2% solubility loss for defatted protein (data obtained following a one-day incubation at standard conditions). Since the defatting procedure yields native albumin (Chen, 1967) and no differences between the defatted and non-defatted proteins could be discerned either by SEC, thiol content or water uptake (data not shown), the difference in stability can be directly attributed to the octanoate.

These data may shed light on rHA's increased susceptibility towards aggregation via thiol-disulfide interchange in the wetted lyophilized state (hydrated at 96% relative humidity) compared to that following further addition of water. If rHA exhibits behavior similar to that of BSA, it is likely that the descending portion of the curve in Fig 4.11 is due to a refolding of a partially denaturated (upon lyophilization) protein, i.e., that the lyophilized, partially unfolded rHA exhibits greater aggregation than the rehydrated protein that has folded back to its native conformation.

This hypothesis explains the stabilization imparted by low-molecular-weight excipients co-lyophilized with rHA. During incubation at standard conditions, such an excipient brings extra water to the vicinity of rHA, resulting in refolding into the native, and more stable, conformation. Such a relatively sharp conformational transition occurring would not contradict the water sorption data for the various sorbitol:rHA lyophilizates (Table 4.4) or the dependency on excipient loading (Fig. 4.7B).

4.7 A Note on the Glass Transition Temperature (T_g) and the Stabilizing Effect of Strongly Water-Sorbing Excipients

It has also been proposed that addition of certain excipients may stabilize proteins by raising T_g, the glass transition temperature (Franks, 1990; Franks *et al.* 1991; Hatley and Franks, 1991; Chang and Randall, 1992). In order to determine whether this is a possible scenario to explain the effect of strongly water-sorbing excipients on rHA's solid-phase stability, we shall consider the T_g of rHA under our standard incubation conditions and the influence of such additives. Although the T_g value of solid rHA is unknown, under the relative humidity routinely used for study (96%) it is highly likely that is well below the incubation temperature (37°C). This is based on the observation that, for most proteins, the transition from glassy to rubbery states occurs at room temperature at about 25-33 g water/100 g dry protein (Hageman, 1992). Since T_g drops precipitously with increasing water content of the protein (as dictated by the G-T equation discussed in Section 2.3.2), under our standard conditions of 96% relative humidity and 37°C (where rHA absorbs nearly 47±7 g water/100 g dry protein) the T_g should be well below 25°C, and thus rHA should be in the rubbery state (once the water has been absorbed by the protein).

Thus, an excipient which raises the T_g of the system (to a level above 37°C) may impart stability by maintaining rHA in a glassy state, thereby inhibiting protein aggregation which occurs only in the rubbery state. However, this is not possible for the case of a strongly water-sorbing excipient, regardless of whether the wetted rHA is in the rubbery or glassy state initially (under the standard conditions in the absence of excipients). This is because the addition of such an excipient, and of the water which it draws into the system during the subsequent incubation, will further decrease the T_g of the system (Franks *et al.*, 1991). If we are in the rubbery regime initially (which is highly likely), the decrease in T_g by a water-absorbing stabilizer will not change this, for our incubation temperature will still be above T_g. If we are in the glassy state initially, then the decrease in T_g would either have no effect on the physical state of the system or cause it to become rubbery (T_g drops below the incubation temperature) thereby destabilizing rHA. Thus, in neither scenario does the presence of a water-sorbing stabilizer cause the physical state of the system to proceed from rubbery to glassy under standard incubation conditions.

4.8 Lyophilization-Induced Structural Rearrangement of rHA and the Stabilizing Effect of Strongly Water-Sorbing Excipients

The refolding hypothesis (Section 4.6.3) starts from the premise that rHA undergoes a structural rearrangement upon lyophilization. However, the extent of the structural rearrangement was not considered. It is important to exclude the possibility that excipients may influence the extent of this unfolding which, in turn, may influence solid-phase stability.

We have already examined rHA's structure when co-lyophilized with dextran. The presence of this excipient, which imparts stability by presumably reducing protein-protein contacts, does not significantly affect rHA's structure. To determine whether the strongly water-sorbing excipients may influence rHA's structure, we measured the IR spectra of rHA co-lyophilized with NaCl (1 g per 6 g of dry protein), one of the most potent stabilizers of rHA against moisture-induced aggregation (curve g in Fig 4.9). The Gaussian deconvolution of the IR spectrum of rHA co-lyophilized with NaCl revealed a secondary structural composition nearly identical to that of the protein lyophilized in the absence of excipient (Table 4.2). Thus, the extent of the structural rearrangement caused by lyophilization was unaffected by NaCl. In this case, it appears that the stabilization was indeed afforded solely by the uptake of water in the vicinity of rHA during the high-humidity incubation, as discussed previously (Section 4.6.3).

4.9 The Effect of the Formulation Methodology on rHA's Structure and Stability in the Solid Phase

The data reveal no correlation between lyophilized rHA's stability against moisture-induced aggregation and its secondary structure in the presence of excipients (namely dextran and NaCl). Another, related issue remaining unresolved is how the mode of dehydration may affect these characteristics. To probe this, solid rHA was prepared by spray drying, rotary evaporation, and acetone precipitation, all from pH 7.3. Note that the thiol (Cys^{34}) contents of these samples were the same (see Sections 3.3.5-3.3.7). In addition, rHA was lyophilized from two other pHs, 4.0 and 9.0. For each sample, both the stability against moisture-induced aggregation (during incubation at standard conditions) and the secondary structure following the dehydration (unincubated) were examined and compared to data obtained for protein lyophilized from pH 7.3. The data obtained reveal an appreciable variation in stability among these samples (Figure 4.12). However, there was no major difference in the extent and nature of their dehydration-induced structural reorganization (Table 4.2), even though dehydration by different modes may result in different protein structures (Desai *et al.*, 1994; Desai and Klibanov, 1995). Among the minor effects observed herein, the most notable was that rHA lyophilized from pH 4.0, which exhibited superior stability against moisture-induced aggregation (Fig. 4.12), had a slightly higher percentage of unordered structure (Table 4.2). All of the dehydrated rHA samples lost some α-helices with concomitant increases in β-sheet and unordered structures compared to the protein in aqueous solution (Table 4.2).

The most likely explanation for the dramatically increased stability of rHA lyophilized from pH 4 vs. that lyophilized from either pH 7.3 or pH 9 is the ionization state of the free thiol of Cys^{34}. When lyophilized from an acidic pH, a greater fraction of Cys^{34} is in the protonated form than at neutral or alkaline pHs (Lewis *et al.*, 1980). This -SH form does not participate in thiol-disulfide interchange (where the thiolate ion is the reactive species (Fig. 2.6). This hypothesis was also put forward to explain the pH-dependence of the moisture-induced aggregation of BSA (Liu *et al.*, 1991).

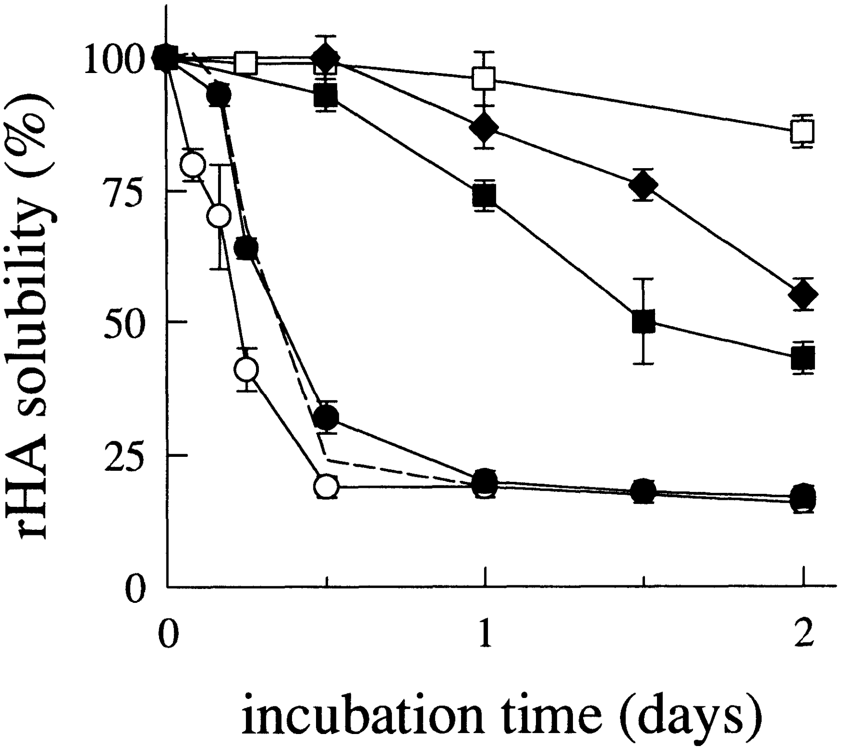


Figure 4.12 Moisture-induced aggregation of various solid rHA formulations. Protein was dehydrated by different methods: [□] lyophilization from pH 4.0; [○] lyophilization from pH 9.0; [●] spray drying from pH 7.3; [■] rotary evaporation from pH 7.3; and [◆] acetone precipitation from pH 7.3. The dashed curve depicts the time course of solubility loss for rHA lyophilized from pH 7.3 (from Fig. 4.2). The thiol (free Cys^{34}) contents of lyophilized, spray-dried, and rotary evaporated rHA (all from pH 7.3) were essentially identical. Incubation conditions are the same as in Fig. 4.2.

A similar rationale provides an explanation for the increased stability of the acetone-precipitated rHA. Since in this case rHA is being removed from the solvent, and not vice versa, it is possible that the ionization state of the precipitated protein is not the same as that in aqueous solution prior to the addition of acetone. For example, the population of protein molecules with an ionization state closer to neutrality (that is, closer to that exhibited at the isoelectric point, pI) may precipitate more readily. To test this idea, acetone-precipitated rHA was reconstituted in water (unbuffered), yielding a pH of 5.8, far lower than the value of 7.3 for the aqueous solution from which it was precipitated. It is noteworthy that the reported pI of human albumin is ~ 5.9 (Ui, 1971; Foster, 1977). In contrast, reconstituted lyophilized rHA yielded a value approximately the same (pH 7.3) as in the aqueous solution prior to freeze drying. Since this reconstitutive approach can be used to indicate solid protein ionization state (Hageman *et al.*, 1992a), it is likely herein that the rHA acetone precipitate has a greater fraction of protonated Cys^{34} species than the lyophilized powder.

This rationale is unlikely to explain to increased stability exhibited by the rotary-evaporated rHA. Another possibility is that due to the different morphology of this preparation (it appeared as a dried film as opposed to the powdery lyophilizate), water uptake rate was slower than that exhibited by the freeze-dried protein. Since water content is linked to aggregation (Section 4.6.2), slower water uptake would lead to decreased aggregation rates. Indeed, the water uptake of the rotary-evaporated rHA after a 24-h standard incubation was 23±2 g/100 g protein, far lower than the some 50 g/100 g sorbed by the lyophilizate under these conditions. In any case, the superior stabilities of the acetone-precipitated and rotary-evaporated samples cannot be explained by conservation of their structures upon dehydration.

Therefore, even though dehydration-induced unfolding may be a prerequisite for rHA's aggregation, it is not the only factor influencing protein stability. Apparently, environmental factors also play a major role.

4.10 Concluding Remarks

In summary, we have investigated the intermolecular thiol-disulfide interchange occurring in solid rHA when incubated under pharmaceutically relevant conditions: 37°C and 96% relative humidity. A number of compounds were tested as potential stabilizing excipients at these conditions. Two distinct modes of stabilization have been uncovered: (1) stabilization by reducing protein-protein contacts (e.g., dextran), and; (2) stabilization afforded by strongly water-sorbing excipients (e.g., sorbitol or NaCl) which act as "sponges", drawing moisture from the humidified air and sharing it with the protein. It was hypothesized that this increased water drives a refolding of the rHA molecule, which was partially and reversibly unfolded upon its lyophilization, as revealed by FTIR spectroscopy. This hypothesis was consistent with the aggregation behavior of rHA as a function of water content in the absence of excipients. The extent of the structural rearrangement is relatively insensitive to the manner in which rHA was dehydrated (lyophilized from different pHs, lyophilized with excipients, rotary evaporated, spray dried) even though the stability of these varied significantly. Our findings demonstrate that excipient-water and protein-protein (rather than excipient-protein) interactions dominate protein stability in wetted lyophilized powders. This conclusion should be useful in inhibiting aggregation in solid protein formulations, and may have repercussions for stabilization against deleterious intramolecular processes as well.

CHAPTER 5

MOISTURE-INDUCED AGGREGATION OF SOLID INSULIN

5.1 Motivation and Overview

Insulin is a well-studied and important pharmaceutical protein which is widely used in treatment of insulin-dependent diabetes (Brange, 1987). The stability of the insulin molecule in various formulations is a serious issue in insulin-dependent diabetes therapy (Brange, 1987; Brange, 1994). In particular, aggregation of insulin is known to lead to severely reduced biological activity and increased immunogenicity (Brange, 1987). In addition, the aggregation of insulin suspended in a polymeric matrix within a controlled-release formulation may lead to lowered release rates (Brown *et al.*, 1986). Thus, we have studied the moisture-induced aggregation of lyophilized insulin. Insulin, unlike the model protein considered in the previous chapter, albumin, does not contain a free thiol residue, although it does have disulfide bonds. Therefore, with insulin one can answer the question: what are potential solid-phase aggregation pathways when disulfides, but not free thiols, are present in the native protein structure?

The experimental approach herein was to expose the solid insulin to accelerated storage conditions (high temperature and humidity) and monitor the loss of solubility, which is a reflection of insoluble aggregate formation in the wetted protein. On the basis of the molecular mechanism which was elucidated for insulin's moisture-induced aggregation, various rational stabilization strategies were formulated and verified.

5.2 Aggregation of Bovine Zn-Insulin Dissolved at Acidic pH

5.2.1 Kinetics of aggregation at accelerated storage conditions. A variety of insulin samples were prepared to test their stability toward aggregation in the solid state. Bovine Zn-insulin was dissolved at 0.05, 0.15 and 1.0 mg/ml. Due to the slow dissolution of crystalline Zn-insulin in water at neutral and alkaline pH, dissolution was accomplished by stirring for 20 min at pH 3.0. Prior to lyophilization, insulin solutions were clarified by filtration and adjusted to pH 7.3. In addition, bovine K-insulin was also prepared and lyophilized from 1 mg/ml, pH 7.3. To simulate accelerated storage conditions, we initially investigated the stability of lyophilized bovine insulin at high relative humidity (96%) and an elevated temperature (50°C). At various time points of this incubation, insulin was tested for its solubility in aqueous solution, relative to that of the unincubated insulin. As seen in Figure 5.1, all insulin samples exhibited significant solubility loss during this incubation, i.e., aggregation. None of the unincubated insulin samples (stored at -20°C) showed any aggregation. Thus, the detrimental process occurred during the high temperature/high humidity incubation of the solid insulin and not during the lyophilization (freezing and drying) process.

In order to make stability comparisons between different samples, formation of insoluble aggregates was measured at fixed conditions and lengths of incubation time. To this end, the insulin samples from Fig. 5.1 were incubated at 50°C and 96% relative humidity for one- and two-week periods. The resulting losses in protein solubility are shown in Figure 5.2. It is seen that there is no clear dependence of the degree of aggregation on insulin concentration prior to lyophilization. However, aggregation appears slightly faster when insulin was lyophilized from lower concentrations, in particular after the 2-week incubation. Such low concentrations presumably result in a lower degree of association of insulin molecules (Milthorpe *et al.*, 1977) which, in solution, results in less stable insulin preparations (Brange, 1987). Under such conditions, insulin molecules may also adsorb at the hydrophobic air-water interface resulting in denaturation and aggregation (Thurow and Geisen, 1984).

5.2.2 Determination of insulin association state by quasielastic light scattering. Various techniques have been employed to analyze the association state of insulin in aqueous solution. (Insulin molecules exist in equilibrium among mono-, di- and hexameric species.) One such method is quasielastic light scattering (Sluzky, 1992) which we have used herein to probe the association state of insulin in aqueous solution prior to

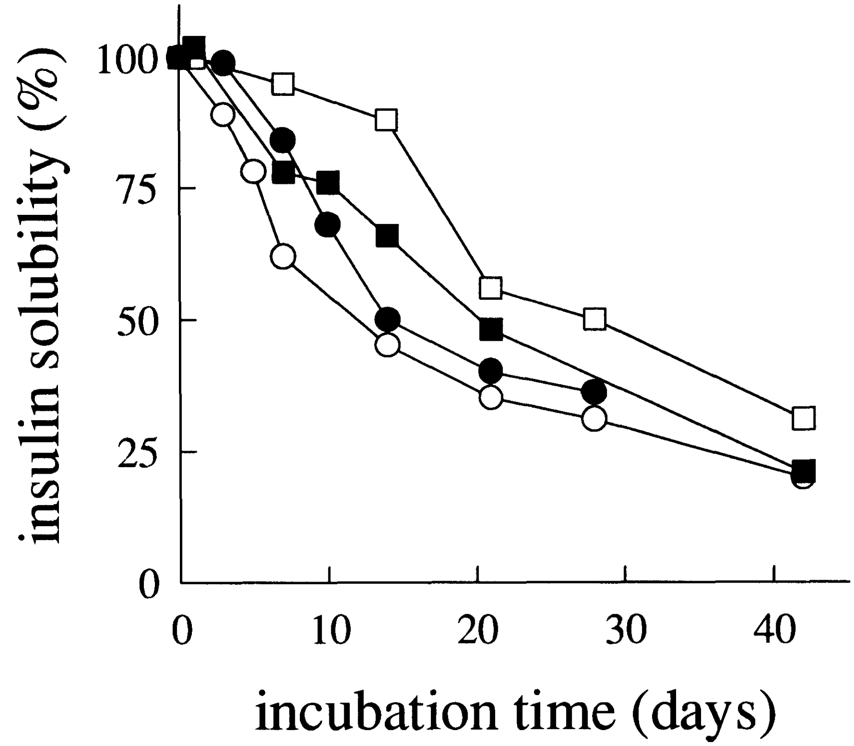


Figure 5.1 Aggregation of lyophilized insulin at 50°C and 96% relative humidity. Zn-insulin was dissolved by stirring in aqueous solution at pH 3.0 for 20 min, and then the pH was adjusted to 7.3. Insulin concentrations prior to lyophilization were: [○] 0.05 mg/ml Zn-insulin, [●] 0.15 mg/ml Zn-insulin, [□] 1.0 mg/ml Zn-insulin, and [■] 1.0 mg/ml K-insulin.

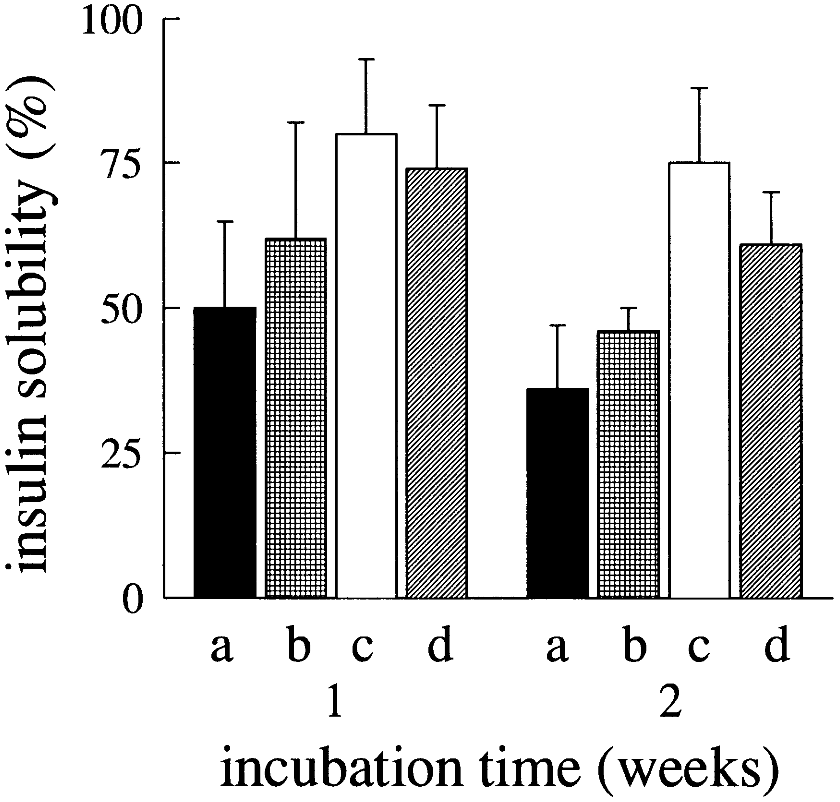


Figure 5.2 Insulin aggregation following one- and two-week incubations at 50°C and 96% relative humidity. Zn-insulin was dissolved by stirring in aqueous solution at pH 3.0 for 20 min, and then the pH was adjusted to 7.3. Insulin concentrations prior to lyophilization were: a, 0.05 mg/ml Zn-insulin; b, 0.15 mg/ml Zn-insulin; c, 1.0 mg/ml Zn-insulin; and d, 1.0 mg/ml K-insulin.

lyophilization (samples from Figs. 5.1 and 5.2). Insulin solutions (all pH 7.3 in water) exhibited the following hydrodynamic radii: 1.0 mg/ml Zn-insulin (Figure 5.3A), 5 nm; 0.15 mg/ml Zn-insulin (B), 1 nm and 5 nm; 0.05 mg/ml Zn-insulin (C), 1 nm; and 1 mg/ml K-insulin (D), 4 nm. (The larger particles (~100 nm) are probably due to soluble, non-covalently bonded aggregates which comprise an extremely small fraction of the insulin molecules (Dathe *et al.*, 1990; Sluzky *et al.*, 1991; Sluzky *et al.*, 1992).) Thus, the sample prepared at 1.0 mg/ml of Zn-insulin consisted of mainly hexamers in solution prior to lyophilization and the sample prepared at 0.05 mg/ml Zn-insulin consisted essentially of only monomers and/or dimers. Both the 0.15 mg/ml Zn-insulin and the 1 mg/ml K-insulin exhibited predominance of monomer/dimer, but also presence of hexamers. The data are consistent with the hypothesis made above that a lower association state prior to lyophilization may result in decreased stability in the solid state.

5.2.3 Elucidation of the aggregation mechanism. In order to investigate the nature of the moisture-induced insulin aggregates obtained, we attempted to re-dissolve the aggregated protein in a number of different solutions. The results obtained are depicted in Table 5.1. In the presence of aqueous solutions of strong denaturing agents, 6 M guanidine hydrochloride and 8 M urea, 56% and 50% of the aggregates dissolved, respectively. This suggests that the aggregates are held together by both covalent and non-covalent forces–the denaturants would disrupt only the latter. When placed in a solution of a thiol reagent, 10 mM dithioerythritol (DTE), 21% of the aggregates dissolved, suggesting that intermolecular disulfide bonds were involved in the insulin aggregate. When both denaturing and reducing agents were present in solution, the aggregates dissolved completely. These data indicate that the insulin molecules in the aggregates are held together by non-covalent interactions and also by intermolecular disulfide linkages.

The formation of covalent and non-covalent insulin aggregates has been observed in other insulin systems. In agitated solutions with hydrophobic interfaces, insulin exhibits non-covalent aggregation initiated by denaturation (Sluzky *et al.*, 1991). Long-term stability studies of various insulin therapeutic preparations (solutions and suspensions) also

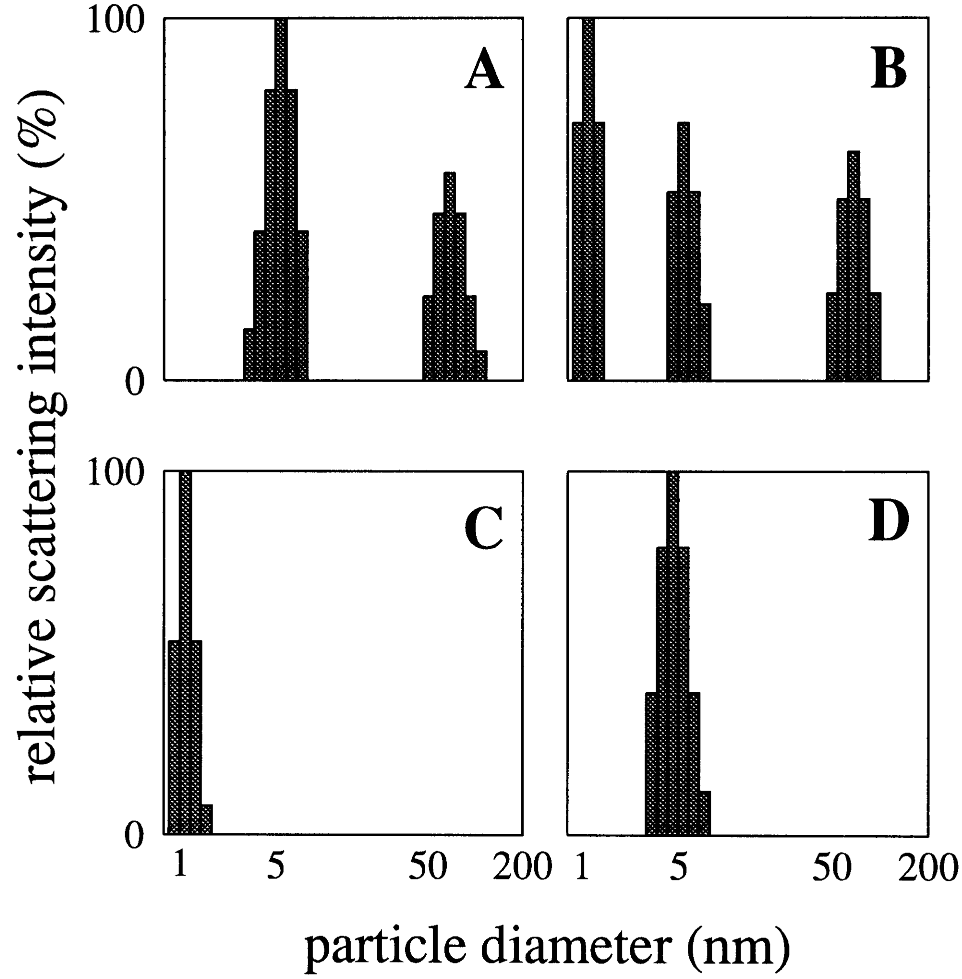


Figure 5.3 Quasielastic light scattering analysis of insulin solutions prior to lyophilization. Zn-insulin was dissolved by stirring in aqueous solution at pH 3.0 for 20 min, and then the pH was adjusted to 7.3. Insulin concentrations prior to lyophilization were: [A] 0.05 mg/ml Zn-insulin, [B] 0.15 mg/ml Zn-insulin, [C] 1.0 mg/ml Zn-insulin, and [D] 1.0 mg/ml K-insulin.

Table 5.1

Solubility of moisture-induced insulin aggregates in various solutions[a]

Solvent	Insulin aggregates solubility (%)[b]
PBS[c]	0[d]
PBS + 1 mM EDTA	0
PBS + 10 mM DTE[e] + 1 mM EDTA	21
PBS + 6 M guanidine hydrochloride	56
PBS + 8 M urea	50
PBS + 8 M urea + 10 mM DTE + 1 mM EDTA	104
PBS + 6 M guanidine hydrochloride + 10 mM DTE + 1 mM EDTA	98

[a] Insulin (lyophilized from 1 mg/ml at pH 7.3) was incubated for 8 weeks at 50°C and 96% relative humidity. After incubation of each 1-mg sample, 5 ml of PBS (phosphate buffered saline: 5 mM sodium phosphate, pH 7.3, and 150 mM NaCl) with or without additional agents was added, and the sample was stirred for 30 min (PBS and PBS + EDTA samples were stirred for 2 h) at 37°C. Insoluble materials were removed by filtration, the filtrate was assayed for protein, and the protein concentration was compared to that in the unincubated insulin.

[b] Note that separate calibration curves were obtained for protein determinations in the presence of the additives listed.

[c] PBS:phosphate-buffered saline.

[d] The amount of aggregates dissolved in these solvents was ≤ 0 (amount of unincubated rHA dissolved).

[e] DTE:dithioerythritol

revealed covalent dimers and soluble oligomers formed over time via intermolecular disulfide bonds (Brange, 1992; Brange and Langkjaer, 1992; Brange *et al.*, 1992). In addition, aggregation of solid, crystalline Zn-insulin has been observed at elevated temperatures due to an undetermined mechanism (Fisher and Porter, 1981).

The formation of intermolecular disulfide bonds requires the presence of free thiols. The insulin monomer has three disulfide bonds (two interchain, one intrachain), but no free cysteines. Hence, an existing disulfide must serve as a precursor of a free thiol. Such an event may occur to the insulin molecule via β-elimination of an intact disulfide (Brange, 1992; Helmerhorst and Stokes, 1983). The mechanism for this process involves a hydroxide ion catalyzing the cleavage of a carbon-sulfur bond resulting in two new residues, dehydroalanine and thiocysteine. The former reacts with lysine to form a lysinoalanine cross-link, and the latter undergoes further decomposition to yield thiol-containing products, such as cysteine and hydrosulfide ion (Fig. 2.4). These free thiols can subsequently catalyze the reshuffling of intact protein disulfides, thereby leading to intermolecular disulfide cross-links (Fig. 2.7). In a study of disulfide exchange in neutral insulin solutions and suspensions, Brange (1992) has suggested that the A^7-B^7 disulfide bridge in the insulin molecule is the most likely to be involved in intermolecular disulfide formation initially (it is the only disulfide near the surface of the folded monomer and not buried upon hexamer formation). Furthermore, it was suggested that initial cleavage of this intact disulfide bridge would lead to unfolding of the insulin molecule, thus facilitating further disulfide interchange (Brange, 1992).

5.2.4 Effect of the pH prior to lyophilization. Because of the involvement of hydroxide ions, β-elimination accelerates under alkaline conditions. Lyophilized protein powders exhibit the ‘pH memory’, namely, in the dry state (or in anhydrous solvents) they exhibit the ionization state and, in turn, behavior corresponding to the pH conditions in aqueous solution prior to lyophilization (Klibanov, 1989). Therefore, one would expect that the covalent aggregation of solid insulin lyophilized from alkaline pH should be faster than that of insulin lyophilized from neutral or acidic conditions. Indeed, for disulfide exchange occurring in insulin solution dramatically increased rates were observed above pH 8.0 (Brange and Langkjaer, 1992). In the present study, a similar trend was observed experimentally for lyophilized insulin, as shown in Figure 5.4. For example, insulin lyophilized at a concentration of 1 mg/ml from pH 10 exhibits complete aggregation in just one day at 50°C and 96% relative humidity, whereas insulin lyophilized at pH 7.3 exhibits less than 50% aggregation under these conditions after incubation for three weeks.

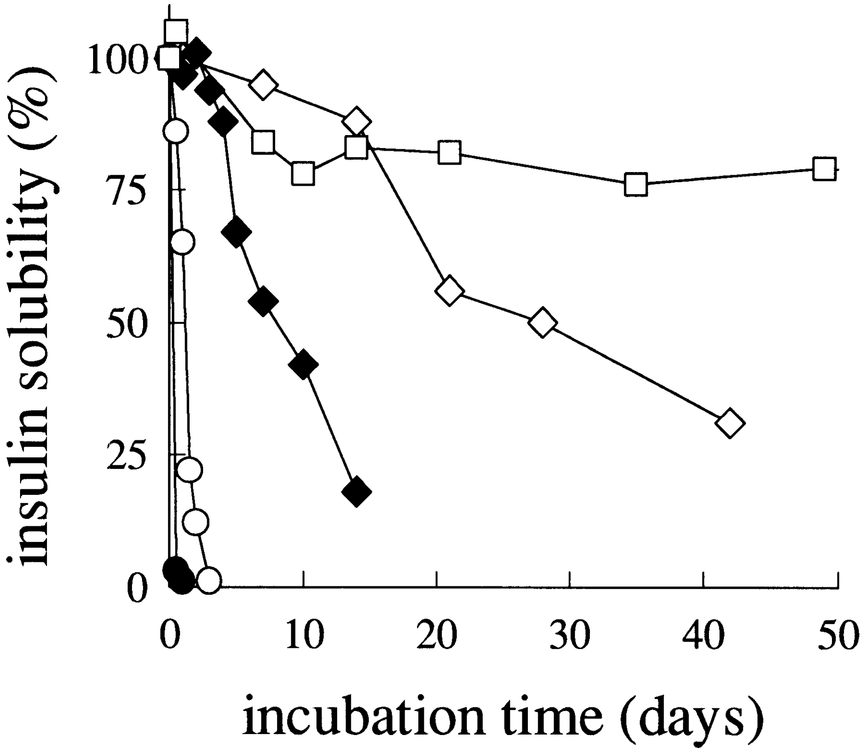


Figure 5.4 Dependence of insulin solid-phase aggregation on the pH of the aqueous solution prior to lyophilization. Insulin was dissolved at 1.0 mg/ml by stirring at pH 3.0 for 20 min, and the pH was subsequently adjusted to varying pH values: [□] pH 3.0, [◊] pH 7.3, [♦] pH 8.0, [○] pH 9.0, and [●] pH 10.0. These samples were incubated at 50°C and 96% relative humidity.

5.3 Aggregation of Bovine Zn-Insulin Dissolved at Alkaline pH (Rapidly Aggregating Insulin)

5.3.1 Kinetics of aggregation at accelerated storage conditions. If the covalent aggregation is initiated by β-elimination, then insulin dissolved at alkaline pH should exhibit increased aggregation due to the formation of free thiols in aqueous solution *prior* to lyophilization (Helmerhorst and Stokes, 1983). This was found to be indeed the case. Insulin dissolved at 0.05 mg/ml and pH 10 (and subsequently adjusted to pH 7.3, from which it was lyophilized) exhibited extremely rapid aggregation at 50°C and 96% relative humidity: a 90% solubility loss was observed following just a 24-h incubation. (Insulin prepared in this manner is henceforth referred to as 'rapidly aggregating' insulin.) This aggregation rate is much faster than that observed for acid-dissolved insulin lyophilized from the same conditions and subjected to the same environment: after one month of incubation the loss of solubility was less than 75% (open circles from Fig. 5.1).

5.3.2 Evidence for formation of free thiols prior to lyophilization. In this comparison, all experimental steps and variables were held constant except for the initial dissolution. Therefore, we conclude that the additional instability exhibited for rapidly aggregating insulin must be the result of an event which transpired during the alkaline dissolution process, presumably the β-elimination reaction. Using Ellman's reagent, DTNB, we indeed confirmed the presence of free thiols in unincubated rapidly aggregating insulin at a level of 0.007±0.003 mol thiol/mol insulin. In contrast, there were no detectable free thiols in the insulin sample prepared by dissolution at acidic pH.

These results demonstrate that different methods of insulin preparation may have a dramatic effect on solid-state stability. This phenomenon may be responsible for the observation that the rates of formation of high molecular weight aggregates in insulin solutions and suspensions varied with source of insulin, as well as with biological activity and composition of the insulin formulation (Brange *et al.*, 1992). Thus, it is important to judiciously select downstream processing conditions prior to storage and delivery of insulin and other pharmaceutical proteins.

5.3.3 Elucidation of aggregation mechanism for rapidly aggregating insulin. In order to understand how different methods of preparation can affect insulin stability in the solid state, we examined the aggregation process for the rapidly aggregating insulin sample mechanistically, using the same approach for acid-dissolved insulin (Table 5.1). The results from the dissolution analysis of the moisture-induced aggregates of rapidly aggregating insulin are shown in Table 5.2. In this case, the addition of aqueous solutions of denaturants did not appreciably dissolve the aggregates, whereas the addition of a thiol-reducing agent resulted in their complete dissolution. Hence, the predominant mode of aggregation of the rapidly aggregating insulin sample is intermolecular disulfide bond formation. Presumably, in this case the rate of aggregation via disulfide formation is so much faster than that of the non-covalent route that the later mechanism is no longer appreciable.

5.3.4 Evidence for formation of free thiols as a consequence of solid-state incubation. To determine whether free thiols formed as a result of a process occurring *in the solid state*, rapidly aggregating insulin was incubated for 24 h at 50°C and 96% relative humidity (resulting in a 95% loss in solubility), and, subsequently, the soluble portion was analyzed by the DTNB assay. The level of free thiols was found to be 0.033±0.004 mol thiol/initial mol of insulin. (This is the average value and standard deviation of four separate determinations using two different samples of rapidly aggregating insulin.) Thus, additional free thiols (0.033 vs. 0.007 mol/mol) indeed formed as a result of the aggregation process during the high temperature/high humidity incubation in the solid state. When this sample was passed through a filter with a molecular weight cutoff of 3000 Da the thiol concentration in the filtrate and the retentate were found to be approximately equal (i.e., the thiol concentration did not increase when the insulin concentration increased). This indicates that the thiols formed during solid-state aggregation were predominantly low molecular weight, perhaps hydrosulfide ion, as opposed to being protein-bound. To our knowledge, these data represent the first direct mechanistic evidence for β-elimination of a protein occurring in the solid state.

Table 5.2

Solubility of moisture-induced aggregates of the rapidly aggregating insulin sample in various solutions[a]

Solvent	Insulin aggregates solubility (%)[b]
PBS[c]	0[d]
PBS + 8 M urea	3 ± 10
PBS + 10 mM DTE[e] + 1 mM EDTA	122 ± 7
PBS + 8 M urea + 10 mM DTE + 1 mM EDTA	110 ± 16

[a] Rapidly aggregating insulin was prepared by lyophilization from an aqueous solution at 0.05 mg/ml and pH 7.3, following dissolution for 2 h at 37°C and pH 10. Samples were incubated for 24 h at 50°C and 96% relative humidity.

[b] Note that separate calibration curves were obtained for protein determinations in the presence of the additives listed.

[c] PBS:phosphate-buffered saline.

[d] The amount of aggregates dissolved in these solvents was ≤ 0 (amount of unincubated rHA dissolved).

[e] DTE:dithioerythritol

5.3.5 Effect of temperature on insulin solid-phase aggregation. It was important to examine the effect of temperature upon the stability of rapidly aggregating insulin. To this end, the latter was incubated at 75% relative humidity and temperatures of 37°C and 45°C, and the data were compared to those obtained at 50°C (Figure 5.5). The rate of insulin aggregation was found to drop precipitously with temperature: aggregation is nearly complete after a 2-day incubation at 50°C, yet the half-time of aggregation is approximately 2 days at 45°C, and more than 3 weeks at 37°C.

The observation that moisture-induced insulin aggregation is significant even at 37°C is of concern regarding its formulations in a controlled-release device. It is essential for therapeutic protein within an implanted polymeric device to maintain consistent pharmaceutical integrity at this temperature for many months. This issue is particularly important with insulin because its levels need to be carefully controlled to avoid serious effects, such as hyperglycemia, which could be fatal (Brange, 1987).

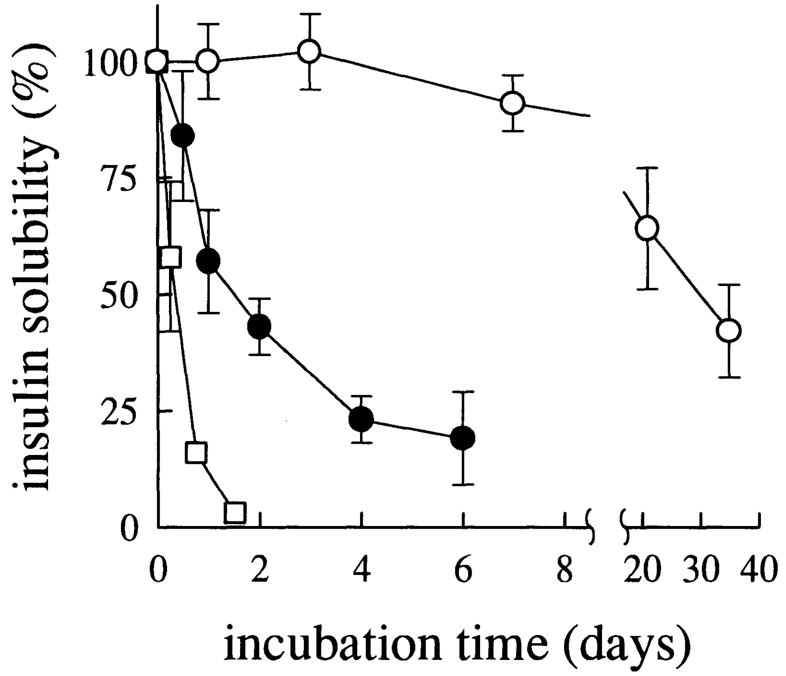


Figure 5.5 Dependence of insulin solid-phase aggregation on the temperature. Rapidly aggregating insulin was prepared and incubated in controlled-humidity chambers containing saturated K_2SO_4 solutions held at [○] 37°C, [●] 45°C and [□] 50°C.

5.4 Development of Rational Stabilization Strategies

5.4.1 Controlling the pH prior to lyophilization. Several rational stabilization strategies may be proposed based on the covalent aggregation mechanism presented above. One approach would be to control the formation of thiolate ions. For instance, if the final pH of the aqueous solution of insulin prior to lyophilization is acidic, one should expect higher stability against the aggregation because (i) pre-formed thiols will not be dissociated (and it is the thiolate ion which is the reactive species in the interchange process) and (ii) subsequent thiol formation during incubation in the solid state will be diminished. This prediction was verified experimentally (Figure 5.6). All samples were prepared in the same manner as rapidly aggregating insulin, except the final pH was varied between pH 4.5 and 8.5. For this experiment, the incubation temperature was lowered to 45°C to slow down the aggregation process in order to distinguish clearly the differences in reaction rates. Compared to insulin lyophilized from pH 8.5 or pH 7.3, insulin lyophilized from pH 6.5 displays a much higher stability in the solid state; furthermore, insulin lyophilized from pH 4.5 is completely stable under the same conditions.

5.4.2 Oxidation of free thiols by transition metal ions. In addition to lowering the formation of thiols, another approach to stabilize insulin is to chemically transform the free thiols to a species non-reactive toward disulfides. Free thiols can be oxidized by molecular oxygen in the presence of certain transition metal ions (Hopton *et al.*, 1968). Consequently, we investigated a number of cations for their potency in stabilizing the rapidly aggregating insulin against moisture-induced aggregation in the solid state. Divalent metal chlorides were added to the insulin preparations just prior to lyophilization in a mol ratio (cation-to-insulin) of 1:10. These samples were incubated for 24 h at 50°C and 96% relative humidity, and the resultant solubility losses were compared with that of insulin prepared without added metal ions (Figure 5.7). As a negative control, $CaCl_2$ was added to one formulation and had no influence on insulin aggregation in the solid state. Thus, any stabilization imparted by other metal salts is due to the presence of the cation and not the chloride ion.

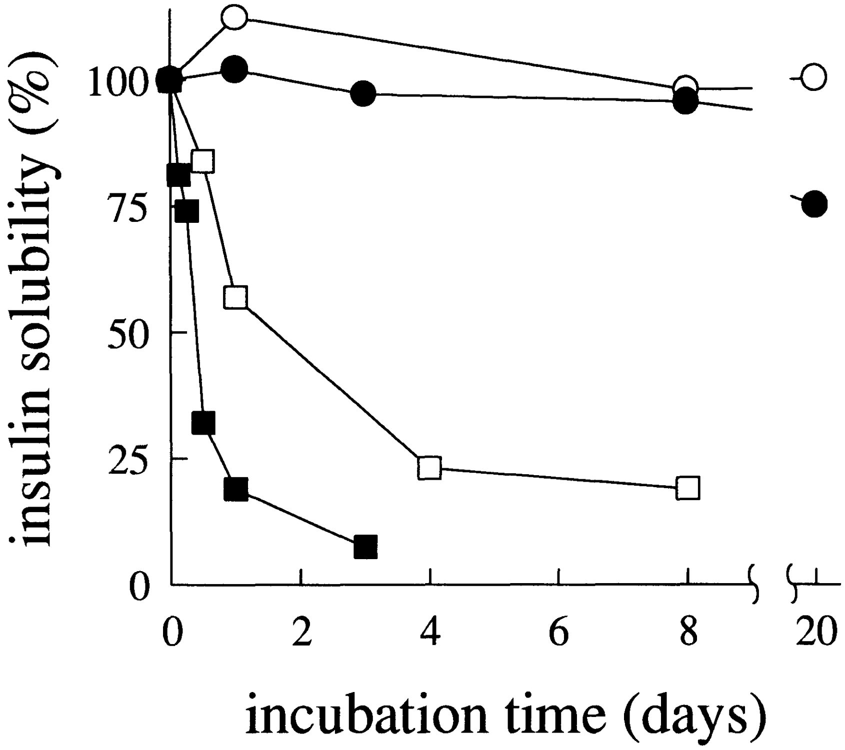


Figure 5.6 Dependence of solid-phase aggregation of rapidly aggregating insulin on the pH of the aqueous solution prior to lyophilization. Rapidly aggregating insulin was prepared and the pH was subsequently adjusted to [O] 4.5, [●] 6.5, [□]7.3, or [■] 8.5 prior to lyophilization. Samples were incubated at 45°C and 75% relative humidity.

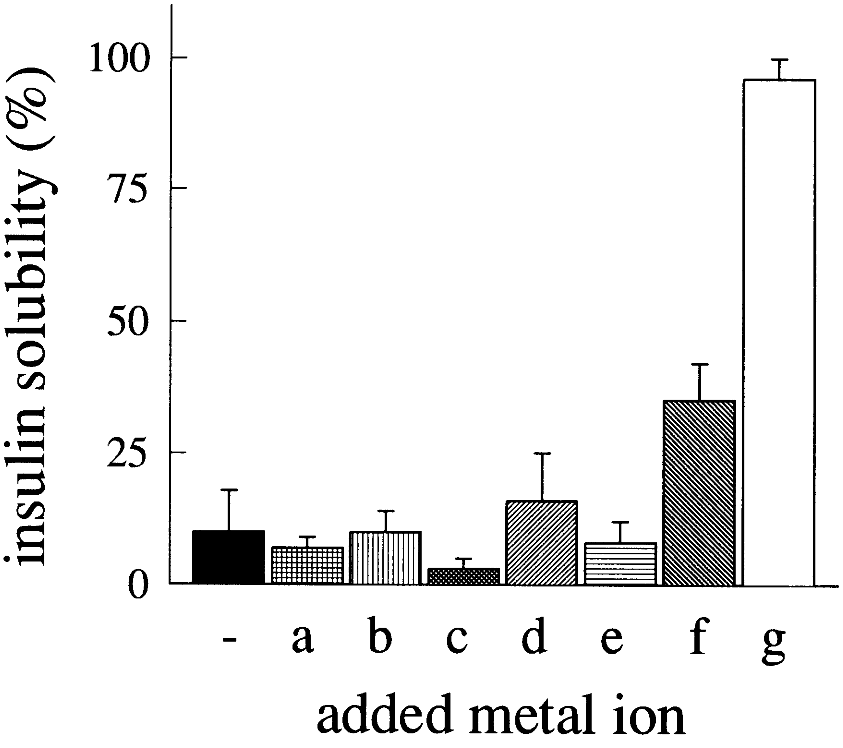


Figure 5.7 Effect of addition of divalent metal ions on solid-state stability of insulin. Rapidly aggregating insulin was prepared in the absence (-) and presence of various metal chlorides: a, Ca^{2+}; b, Fe^{2+}; c, Fe^{3+}; d, Mn^{2+}; e, Ni^{2+}, f, Co^{2+}; g, Cu^{2+}. The metal ion to insulin mole ratio was 1:10. Depicted is the insulin solubility remaining following a 24-h incubation at 50°C and 96% relative humidity.

Although all of the transition metal ions investigated are capable of catalyzing the oxygen oxidation of thiols, only Cu^{2+}, and to a lesser extent Co^{2+}, were effective in significantly stabilizing the rapidly aggregating insulin. In fact, the presence of cupric ion was sufficient to completely stabilize the rapidly aggregating insulin against formation of insoluble aggregates under the conditions studied.

Investigations of the catalytic oxidation of model thiols in aqueous solution have shown that of the variety of metal ions tested, Cu^{2+}, Co^{2+}, and Ni^{2+}, are by far the most effective in term of rates of catalysis, and that cupric ion is the foremost of these (Hopton *et al.*, 1968). Presumably, Cu^{2+}, and to a lesser extent Co^{2+}, were capable of catalyzing the oxidation of thiols at a rapid rate relative to that of thiol-catalyzed disulfide interchange. For the other transition metal ions the relative oxidation rates must have been slower, and hence no significant effect on aggregation was observed.

5.4.3 Controlling the water activity during solid-phase incubation. Yet another approach to stabilization of insulin in the solid state is controlling the humidity. This line of investigation may also help elucidate the role of moisture in solid-state aggregation. Previous investigations have demonstrated that increasing the amount of sorbed water on solid proteins results in increased rates of intermolecular protein reactions (Hageman, 1988; Hageman, 1992; Costantino *et al.*, 1994b). It follows that if water is involved in these processes as a reactant, as a mobile phase for reactants, or as a molecular lubricant (Hageman, 1988; Hageman, 1992), then storage of lyophilized insulin at lower relative humidity (resulting in less protein-bound water) will result in greater solid-state stability. This prediction was confirmed experimentally (Figure 5.8). Rapidly aggregating insulin was incubated at 50°C and a number of relative humidities ranging from 11% to 96%. At the lowest humidity (11%), insulin exhibited complete stability towards moisture-induced aggregation. As the humidity was raised to 65%, there was slight aggregation in the wetted powder. The fastest formation of insoluble aggregates was observed at relative humidities of 75% and above.

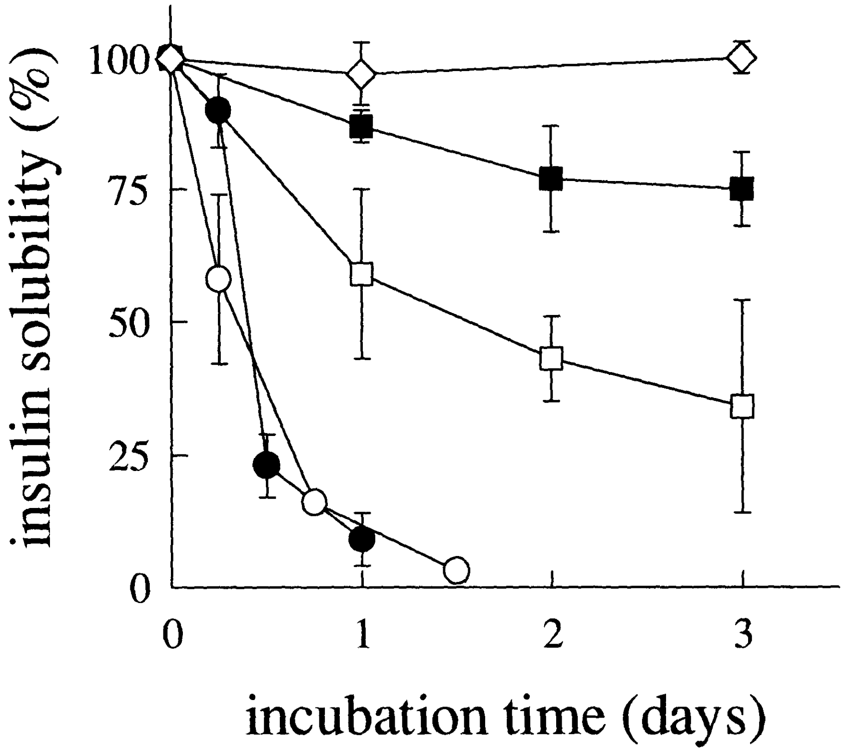


Figure 5.8 Solid-phase aggregation of lyophilized insulin as a function of relative humidity. Rapidly aggregating insulin was prepared and incubated at 50°C and various humidities: [●] 96%; [○] 75% rh; [□] 69%; [■] 65%; and [◊] 11% rh.

5.5 Effect of Moisture on Insulin Solid-Phase Aggregation

5.5.1 Relationship between insulin solid-phase aggregation and moisture content. Of course, the relative humidity itself is not the ultimate factor influencing insulin's stability; instead, it is the water that is sorbed directly on the lyophilized powder. To explore in more detail the relationship between insulin aggregation and the amount of protein-bound water in the solid state, we determined the water vapor sorption isotherm for the rapidly aggregating insulin sample. The data, depicted in Figure 5.9A, indicate that incubation at relative humidities up to 65% results in minimal water uptake by the lyophilized powder. (The same behavior was observed for the insulin water sorption isotherm at 25°C.) Average water content of all samples following incubation at 11% through 65% relative humidity is 6.2±1.7 g/100 g dry protein, i.e., equivalent to the water content of the unincubated sample (5.3±0.9). Thus, at relative humidities up to 65%, little additional water was sorbed beyond that already present in the lyophilized sample. However, above 65% the lyophilized insulin powder exhibited significantly enhanced water sorption. Importantly, it is in this range where insulin aggregation greatly accelerates (Figure 5.9B). Thus, as predicted, the aggregation rate rose markedly with increasing sorbed water.

5.5.2 Modeling the insulin water sorption isotherm and predicting monolayer water coverage. The water sorption isotherm for insulin was modeled using the Brunauer-Emmett-Teller (BET) (1938) equation, as depicted by the solid curve in Fig. 5.9A. The BET isotherm has been extensively used for proteins (Hsu *et al.*, 1991; Hageman *et al.*, 1992b; Schnepf, 1989) and our experimental data for insulin exhibited a fair fit to this model (the correlation coefficient was 0.84 for the BET plot). The BET model was useful in calculating a theoretical monolayer water coverage of the insulin molecule, M_0=4.6 g/100 g protein. In reality, this water exists not as a contiguous monolayer surrounding the protein surface, but is clustered at the charged/polar sites on the protein surface (Rupley *et al.*, 1983). Note that according to the aggregation data (Fig. 5.9B), little or no aggregation was observed at or below insulin monolayer water sorption, in accordance with general rules of solid-phase stability (Hageman, 1988; Hageman, 1992).

The monolayer water coverage was also estimated using a hypothesis proposed by Pauling and Green (Pauling, 1945; Green, 1948) which predicts water sorption monolayers in good agreement with experimental data (Hsu *et al.*, 1991). According to this hypothesis, protein side chains can be divided into strong water-binding sites (strongly polar), weak water-binding sites (weakly polar), and sites with negligible water-binding capacity (non-polar). Any additional water sorbed on the protein forms multilayers. This model assumes that in a low-moisture environment, one molecule of water is sorbed to each strong water-binding site, and no other sites sorb water.

The bovine insulin molecule contains a total of 14 strong water-binding residues: 4 Glu, 4 Tyr, 3 Ser, one Thr and the carboxy termini of the A- and B-chains (Table 5.3). Accordingly, the hypothesis predicts 14 mole-equivalents of water bound to strong water-binding sites which corresponds to 4.6 g/100 g dry protein at a monolayer coverage. This is the same exact value obtained from the BET plot. Taking the Pauling and Green hypothesis a step further, one can examine the weak water-binding residues (weakly polar) in the insulin molecule: 3 Asn, 3 Gln, 2 His, one Lys, one Arg, and the amino termini of the A- and B-chains (Table 5.3). Accordingly, if all strong and weak water-binding sites were occupied, the water content would be 8.5 g/100 g dry protein. Because the BET results are in such good agreement with the theoretical calculation employing only strongly water-binding site, it is likely that the weakly polar and non-polar groups do not contribute significantly to the formation of the insulin's water monolayer. Since the water content of the insulin used to determined the sorption isotherm in Fig. 5.9A was 5.3±0.9 g/100 g dry protein, we conclude that all the strong water-binding sites and some of the weak ones contained sorbed water initially. It follows then that any additional sorbed water would form multilayers, a conjecture which leads to a water sorption isotherm where multilayer water sorption dominates, which is consistent with the observed data (Fig. 5.9A).

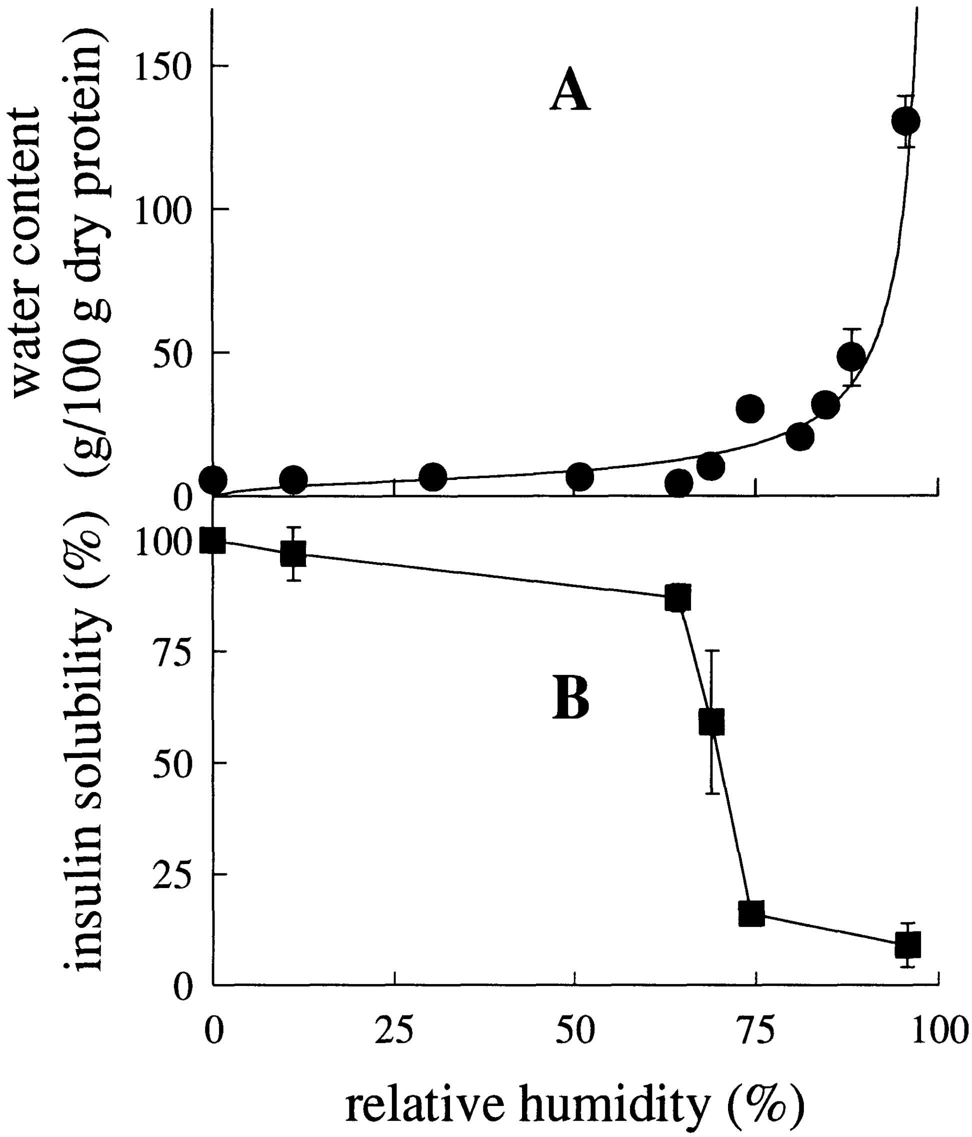


Figure 5.9 Lyophilized insulin aggregation as a function of water sorption of the protein. The top figure (A) depicts the sorption isotherm for the rapidly aggregating insulin sample determined at 50°C. The solid curve represents the fit to the Brunauer-Emmett-Teller (BET) equation with the parameters M_0=4.6 g water/100 g protein and C=13.6. The lower figure (B) depicts the aggregation of fast aggregating insulin following incubation for 24 h at 50°C at various relative humidities.

Table 5.3

Strong and weak water-binding sites on the insulin monomer according to the hypothesis of Pauling and Green[a]

Insulin strong water-binding sites		***Insulin weak water-binding sites***	
Amino acid	Groups per insulin monomer	Amino acid	Groups per insulin monomer
Asp	0	Trp	0
Glu	4	His	2
Ser	3	Lys	1
Thr	1	Arg	1
Tyr	4	Asn	3
-COOH term.	2	Gln	3
		-NH_2 term	2
Total strong water-binding sites:[b] 14		*Total weak water-binding sites:*[c] 12	

[a] Approach taken from Pauling (1945) and Green (1948).

[b] Water content if all of the strong water-binding sites were sorbed would be 4.6 g/100 g dry protein.

[c] Water content if all of the strong and weak water-binding sites were sorbed: 8.5 g/100 g dry protein.

5.6 Concluding Remarks

We have also investigated the behavior of solid recombinant human insulin, a common source of insulin for therapeutic use (Brange, 1987). Samples of Zn-free human insulin were prepared by dissolution under acidic conditions (stirring at pH 3.0 for 20 min) and then adjusting the final pH to 6.5, 7.3, or 8.5. These samples were lyophilized and incubated at 50°C and 96% relative humidity. The solubility loss of these samples is shown in Figure 5.10. The data demonstrate a pattern of aggregation similar to those of bovine insulin samples: aggregation rate increases with pH prior to lyophilization. Considering the structural similarity of bovine and human insulin, which differ by only three amino acid residues (Brange, 1987), it is reasonable to assume that human insulin undergoes the same mechanisms of aggregation at its bovine counterpart.

In summary, the formation of insoluble aggregates in lyophilized insulin at elevated temperature and high humidity has been investigated and mechanistically rationalized for the first time. The moisture content of the lyophilized protein was found to directly correspond with its instability, signifying the protein conformational mobility plays a significant role in aggregation. The aggregates are formed via two distinct mechanisms which occur in the solid state: non-covalent interactions and β-elimination of cystines, followed by thiol-catalyzed disulfide interchange. On the basis of our findings, rational approaches for stabilization have been proposed and verified including controlling pH and the formation/transformation of reactive thiolate ions. These findings may be useful to the development of sustained-release formulations of insulin and other pharmaceutical proteins.

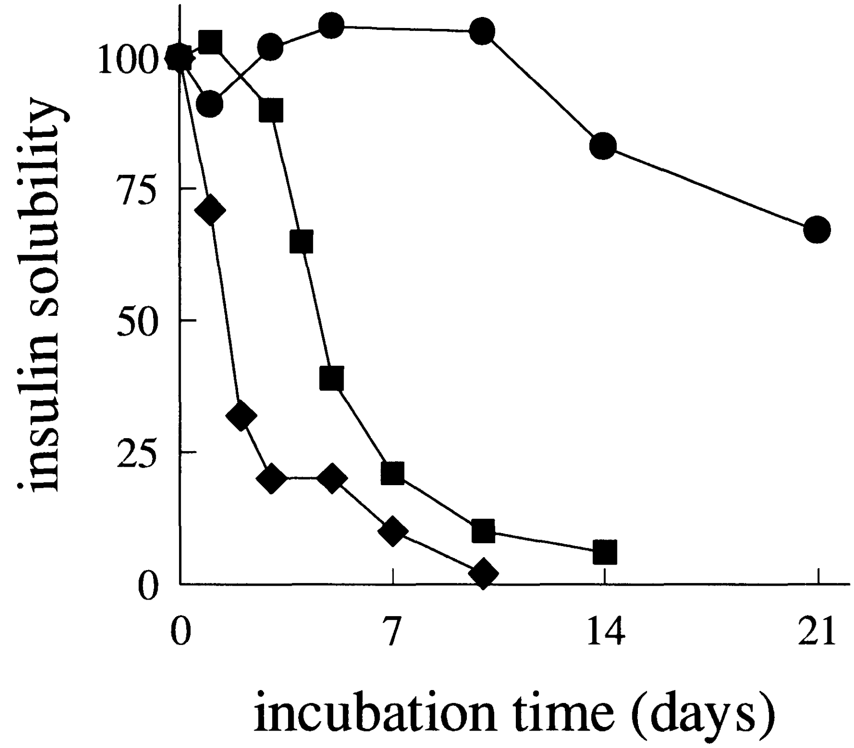


Figure 5.10 Aggregation of lyophilized Zn-free human insulin at 50°C and 96% relative humidity. Insulin was dissolved at 1.0 mg/ml by stirring in aqueous solution at pH 3.0 for 20 min, and the pH was subsequently adjusted to [●] 6.5, [■] 7.3, and [◆] 8.5 prior to lyophilization.

CHAPTER 6

MOISTURE-INDUCED AGGREGATION OF SOLID TETANUS TOXOID

6.1 Motivation and Overview

Vaccine proteins comprise an important group of biopharmaceuticals. These agents, such as tetanus toxoid (TT) and diphtheria toxoid (DT), can be highly successful in immunization and prevention of diseases. Even so, neonatal tetanus remains a major cause of death in developing countries (Aguado and Lambert, 1992) and diphtheria has recently become an epidemic even in some developed countries (Maurice, 1995a, 1995b). This is primarily due to the failure to administer additional boosters following the initial dose (Aguado and Lambert, 1992). Therefore, protein vaccines are candidates for sustained release from polymers (Esparza and Kissel, 1992; Alonso *et al.*, 1993, 1994; Men *et al.*, 1995). However, incomplete release of TT from one such system, poly[lactic-*co*-glycolic acid] microspheres, indicates that protein vaccines may be prone to solid-phase aggregation (Alonso *et al.*, 1994).

For these reasons, TT is another suitable model for investigation of solid-phase protein stability. The tetanus toxin, like albumin, contains both free thiols and disulfide bonds in the native structure (Bizzini *et al.*, 1977; Eisel *et al.*, 1986). One of these disulfides links the two subunits of TT together, the H- and L-chains (Eisel *et al.*, 1986). However, because TT, like other protein vaccines, is formalinized (detoxified by a 3-week incubation at 37°C in 0.13% (w/w) aqueous formaldehyde at pH 7.6 (Bizzini, 1984)), these groups may be modified. The purpose of this study is to examine how these groups, or others, may be involved in TT solid-phase aggregation. Since the reactivity of these groups depends on their accessibility, it is also relevant to examine the relationship between TT's stability and structural integrity in the solid form. Building upon such observations, various rational stabilization strategies were proposed and evaluated.

6.2 Lyophilization of TT and Perturbations in Secondary Structure

TT, initially obtained as an aqueous formulation, was dialyzed prior to lyophilization. In order to investigate whether the process of freeze drying resulted in any structural changes, we measured the IR spectrum in the amide III region of TT in both aqueous solution (Figure 6.1A) and in the lyophilized form (Figure 6.1B). As was the case for rHA (Fig. 4.1) , the vibrational spectrum of TT undergoes significant changes upon dehydration. To interpret these differences, we again used the approach presented earlier for rHA (Section 4.2), namely Gaussian curve-fitting of the amide III region and assignment of bands to secondary structural elements. The resulting peak wavenumbers, relative areas, and structural assignments are listed in Table 6.1 (and summarized in Table 6.2). For TT's vibrational spectrum, peak wavenumbers were determined by Fourier self-deconvolution of the original spectra, and were in good agreement with the Gaussian curve-fitting results (Table 6.1).

In aqueous 10 mM sodium phosphate pH 7.3, the secondary structure of TT consisted of some 28% α-helix and 46% β-sheet, with the remainder being unordered (Table 6.2). This is in good agreement with the secondary structure of tetanus toxin (the non-toxoided protein) in solution as determined previously by FTIR (Singh *et al.*, 1990; Singh *et al.*, 1993; Fu *et al.*, 1994) and circular dichroism (Singh *et al.*, 1990). (The procedure used to detoxify the tetanus toxin does not result in any major secondary structural changes.) Upon lyophilization, the α-helices decreased to 12% and β-sheets rose to 60%. The percentage of unordered structures was approximately the same in solution and the solid form. Furthermore, we similarly analyzed the secondary structure of TT lyophilizate redissolved in water (at the same pH prior to lyophilization). The data indicate that the structural rearrangement incurred upon lyophilization was largely reversible; the only effect of note was a slight decrease in the percentage of α-helices in the redissolved protein.

Therefore, TT exhibits a dehydration-induced transition from α-helices to β-sheets. This finding is distinct from that presented earlier for rHA, where lyophilization resulted in a transition from α-helices to β-sheets and unordered structures (Section 4.2).

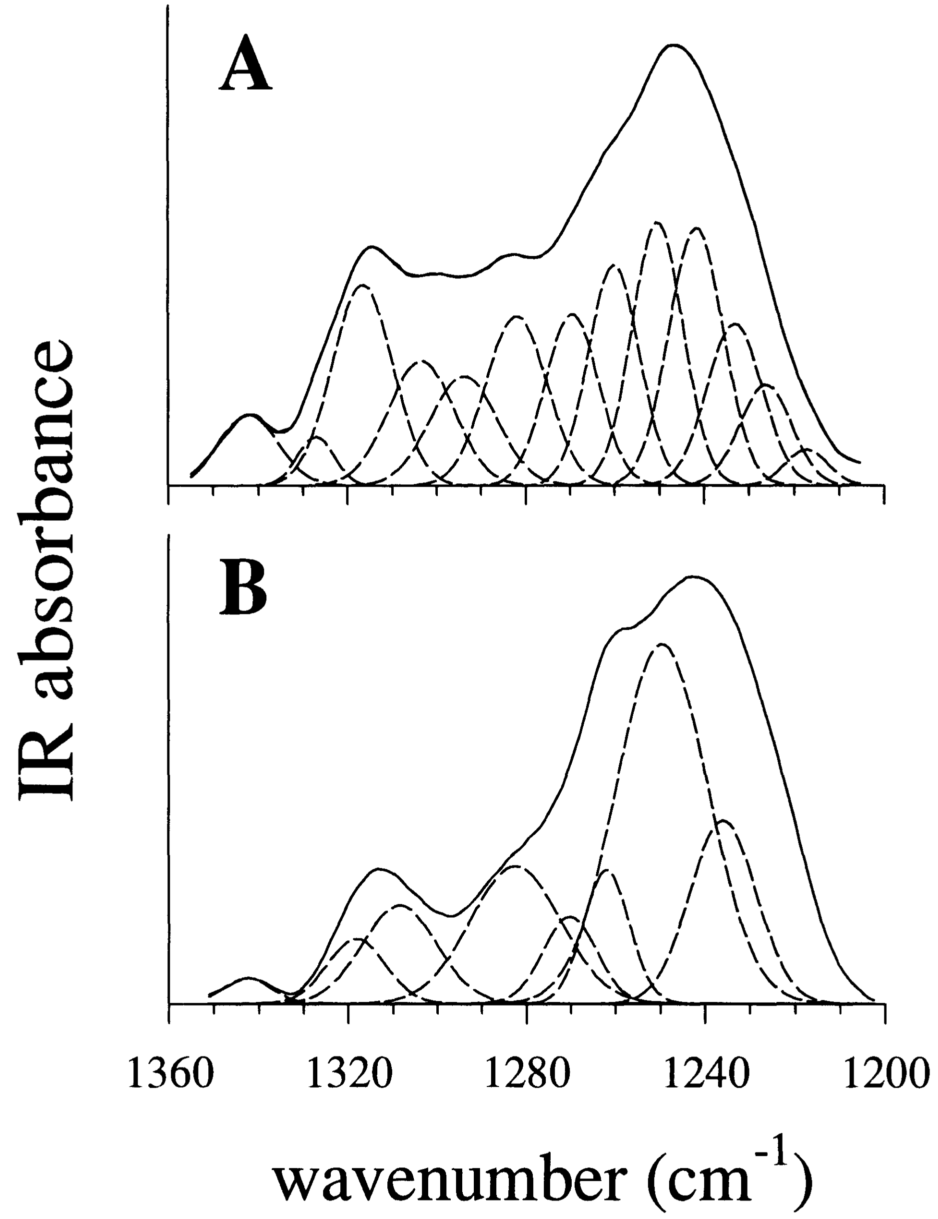


Figure 6.1 IR spectra of TT in the amide III region and their Gaussian curve-fitting. (A) TT dissolved in aqueous solution, pH 7.3; (B) TT powder lyophilized from pH 7.3. The solid curves represent the superimposed original spectra and the Gaussian fit, and the dashed curves are individual Gaussian bands.

Table 6.1

Results of Gaussian curve-fitting of IR spectra in the amide III region for aqueous and lyophilized TT[a]

Aqueous solution[b]				**Lyophilized powder**[c]			
Peak position (cm^{-1})		area		Peak position (cm^{-1})		area	
FSD[d]	GCF[e]	(%)	Assignment	FSD	GCF	(%)	Assignment
	1326±2	4±3	α-helix				
1317±4	1315±2	12±2	α-helix	1314±0	1318±1	5±0	α-helix
1305±4	1304±3	6±2	α-helix	1304±1	1308±0	7±1	α-helix
1289±6	1294±2	6±3	α-helix				
1280±6	1282±1	9±2	coil	1284±1	1281±1	16±1	coil
1268±1	1269±1	6±3	coil	1275±1	1272±0	2±1	coil
1259±2	1260±1	11±1	coil	1261±1	1263±1	10±1	coil
1246±2	1250±1	12±1	β-sheet	1245±1	1249±1	32±2	β-sheet
1238±3	1241±1	17±4	β-sheet	1236±1	1236±1	14±1	β-sheet
	1229±4	10±3	β-sheet	1220±1	1224±1	14±1	β-sheet
1222±3	1226±3	7±4	β-sheet				

[a] Peak locations were initially identified by examination of the Fourier self-deconvolution of the original TT spectra. Assignments of secondary structure was according to Singh *et al.* (1990,1993) and Fu *et al.* (1994).

[b] The aqueous solution contained 1 mM sodium phosphate, pH 7.3.

[c] The pH in aqueous solution prior to lyophilization was 7.3.

[d] FSD:Fourier self-deconvolution.

[e] GCF:Gaussian curve-fitting.

Table 6.2

Secondary structures of various TT formulations as determined by Gaussian curve-fitting of IR spectra in the amide III region

Sample	α-Helix (%)	β-Sheet (%)	Unordered (%)
Aqueous solution[a,b]	28±4	46±6	26±5
Lyophilized (without excipients)[c]	12±1	60±1	28±1
Co-lyophilized with excipients:[c]			
NaCl, 1 g:4 g protein	8±1	68±1	24±1
sorbitol, 1 g:4 g protein	16±2	60±2	24±1
PEG, 1 g:4 g protein	17±2	52±3	31±1
Lyophilizate redissolved in water, pH 7.3 (lyophilized without excipients)	21±1	47±3	33±3

[a] The aqueous solution contained 1 mM sodium phosphate, pH 7.3.

[b] The calculated secondary structural composition in aqueous solution is in good agreement with CD data (Singh *et al.*, 1990) and previous FTIR studies (Singh *et al.*, 1993; Fu *et al.*, 1994).

[c] All lyophilizations were conducted from 1 mM sodium phosphate, pH 7.3.

Whereas rHA's overall structural order decreased upon lyophilization (Section 4.6.3), TT undergoes a reorganization of ordered structures from α-helix and 46% β-sheet. The latter behavior is the more characteristic one; Griebenow and Klibanov (1995) have studied a wide range of proteins and found that, with one exception (i.e., rHA) they all exhibit an increase in structural order upon lyophilization.

6.3 Effect of Moisture on TT Solid-Phase Stability

The intent was to test the solid-state stability of TT under conditions relevant to its release from polymer microspheres. Although there are multiple stages from encapsulation to release wherein TT may deteriorate within solid polymer microspheres (Schwendeman *et al.*, 1995a), the focus herein was to study the rehydration step and the subsequent exposure to moisture experienced by the encapsulated protein. Thus, we chose to incubate solid (lyophilized) TT at 37°C and a wide range of relative humidities and monitor for solubility loss, a reflection of aggregation occurring in the wetted powder.

6.3.1 Relationship between TT solid-phase aggregation and moisture content. Figure 6.2A depicts the solubility loss (i.e., aggregation) of lyophilized TT following a 10-day incubation at 37°C and various levels of relative humidity (rh). The amount of water sorbed on the lyophilizate at these conditions is shown in Figure 6.2B. At low rh (11% rh), which corresponds to negligible water uptake in the protein powder (4±2 g water/100 g dry protein), TT was completely stable. When the rh was raised to 54% there was a small increase in the amount of sorbed water (9±2 g/100g protein) and some minor solid-phase aggregation (16±4% solubility loss). At humidities of 67-90%, the extent of TT solid-phase aggregation rose dramatically (to about 70-90%) as did its water sorption. As the rh was further raised (to 96%), aggregation declined significantly (to 33±4%), while the water sorption continued to increase.

This dependency of TT solid-phase aggregation on water content, that is, its bell-shaped nature, is qualitatively similar to that exhibited by BSA (Liu *et al.*, 1991; Jordan *et al.*, 1994) and rHA (Section 4.6.2). As in those instances, the likely role of water in

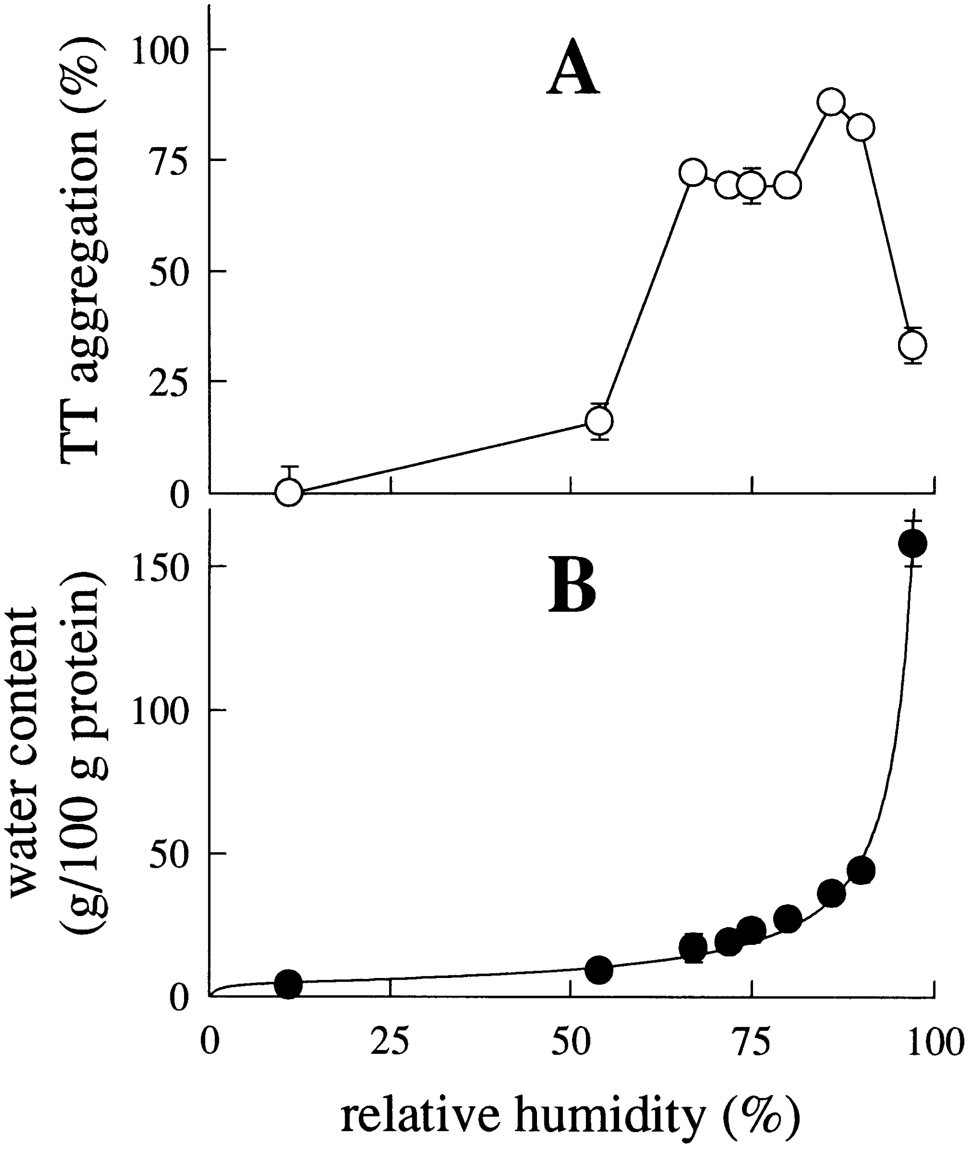


Figure 6.2 Solid-phase aggregation of TT as a function of water sorption of the protein. The top figure (A) depicts the aggregation (solubility loss) of lyophilized TT following a 10-day incubation at 37°C and various relative humidities. The lower figure (B) depicts the sorption isotherm for lyophilized TT at 37°C. The solid curve represents the fit to the Brunauer-Emmett-Teller (BET) equation with the parameters: M_0=4.8 g water/100 g protein and C=86.

destabilizing TT is that it increases protein flexibility which promotes cross-linking (Section 2.4.2). The explanation for the decreased extent of aggregation observed at high rh (96%) is probably due to either a structural transition upon rehydration, which leads to decreased exposure of reactive groups (as was hypothesized for rHA (Section 4.6.3)) or the "dilution" effect (Liu *et al.*, 1991).

6.3.2 Modeling the TT water sorption isotherm and predicting monolayer water coverage. The solid curve in Fig 6.2B represents the BET fit (Brunauer *et al.*, 1938) of TT's water sorption isotherm. This model successfully described the data: the correlation coefficient was 0.91 for the BET plot. From the fit, the theoretical water monolayer on TT was calculated to be 4.8 g/100 g dry protein. The monolayer water coverage for TT was also independently predicted from the known amino acid sequences of the native tetanus toxin (Eisel *et al.*, 1986) using the hypothesis of Pauling (1945) and Green (1958). From the 411 strong water-binding sites on the tetanus toxin molecule (shown in Table 6.3), assuming that each one binds one mol water at monolayer coverage, the predicted value is 4.9 g/100 g protein. This is in excellent agreement with the result from TT's BET plot. Note that according to the aggregation data (Fig. 6.2A), little or no aggregation was observed at or below TT monolayer water sorption, in accordance with general rules of solid-phase stability (Hageman, 1988; Hageman, 1992).

6.4 Mechanisms of TT Solid-Phase Aggregation

At very low rh (e.g., 11%), aggregation of TT is so slow that further study is not practical. However, at higher rh the rates are amenable to mechanistic analysis. Thus, we chose to investigate the mechanism of TT aggregation at two conditions (both at 37°C): (i) 80% rh, corresponding roughly to maximum extent of aggregation (Fig 6.2A) and where the water uptake was 27±3 g/100 g protein (Fig. 6.2B); and, (ii) 96% rh, corresponding to the descending portion of the curve (Fig 6.2A) and where the water uptake was 158±8 g/100g protein (Fig 6.2B).

Table 6.3

Strong water-binding sites on the tetanus toxin molecule according to the hypothesis of Pauling and Green[a]

Amino acid	Groups per TT molecule
Asp	88
Glu	70
Ser	99
Thr	73
Tyr	79
-COOH term	2
Total number of sites:[b] 411	

[a] Approach taken from Pauling (1945) and Green (1948).

[b] Water content if all of the strong water-binding sites were sorbed would be 4.9 g/100 g dry protein.

6.4.1 Mechanistic investigation at intermediate relative humidity. To investigate the mechanism of solid TT aggregation at intermediate humidity, protein aggregates were obtained following a 10-day incubation at 37°C and 80% rh. These aggregates were then reconstituted in various aqueous solutions in order to gain clues about the chemical nature of their cross-links (Table 6.4). Neither the presence of a denaturant (urea) nor a reducing agent, dithiothreitol (DTT) was capable of significantly solubilizing the TT aggregates. These data indicate that aggregation under these conditions was the result of covalent, non-disulfide bonds.

6.4.2 Mechanistic investigation at high relative humidity. The methodology for examining the mechanism of TT aggregation at high humidity was the same as that described in the previous section, except that aggregates were obtained by incubation at 37°C and 96% rh. Under either denaturing or reducing conditions (presence of either urea or DTT), these aggregates were partially solubilized (Table 6.4). These data indicate that TT aggregates have a mixed non-covalent and disulfide-bonded nature. When both the denaturing and the thiol agents were present, TT was again only partially solubilized, indicating that a third aggregation pathway, resulting in covalent, non-disulfide cross-links, had occurred (Table 6.4).

6.4.3 Amino acid analysis of aggregates. To further explore this third, previously undescribed, mechanism of moisture-induced aggregation we examined the amino acid composition for TT aggregates. The incubation conditions chosen were 37°C and 86%, roughly corresponding to the aggregation maximum and representing a value between those two selected for dissolution analysis described above. We subsequently chose these conditions as the standard ones for all additional studies.

The amino acid analysis data are presented in Figure 6.3, for which unincubated TT was used as the control. There was a significant increase in the tyrosine (Tyr) content of the moisture-induced aggregates compared to the unincubated control protein. Furthermore, there was a significant loss of lysine (Lys) and histidine (His) residues in the aggregates relative to the control. (There was also a significant loss of methionine (Met)

Table 6.4

Solubility of moisture-induced aggregates of TT in various solutions[a]

Following incubation at intermediate relative humidity (80%)

Solvent	TT aggregates solubility (%)[b]
PB[c]	0[d]
PB + 6 M urea	0
PB + 10 mM DTT[e] + 1 mM EDTA	0
PB + 6 M urea + 10 mM DTT + 1 mM EDTA	8 ± 3

Following incubation at high relative humidity (96%)

Solvent	TT aggregates solubility (%)[b]
PB	0
PB + 6 M urea	48 ± 8
PB + 10 mM DTT + 1 mM EDTA	20 ± 5
PB + 6 M urea + 10 mM DTT + 1 mM EDTA	55 ± 2

[a] TT was prepared by lyophilization from an aqueous solution at 1 mg/ml and pH 7.3. Samples were incubated for 10 days at 37°C and the rh indicated.

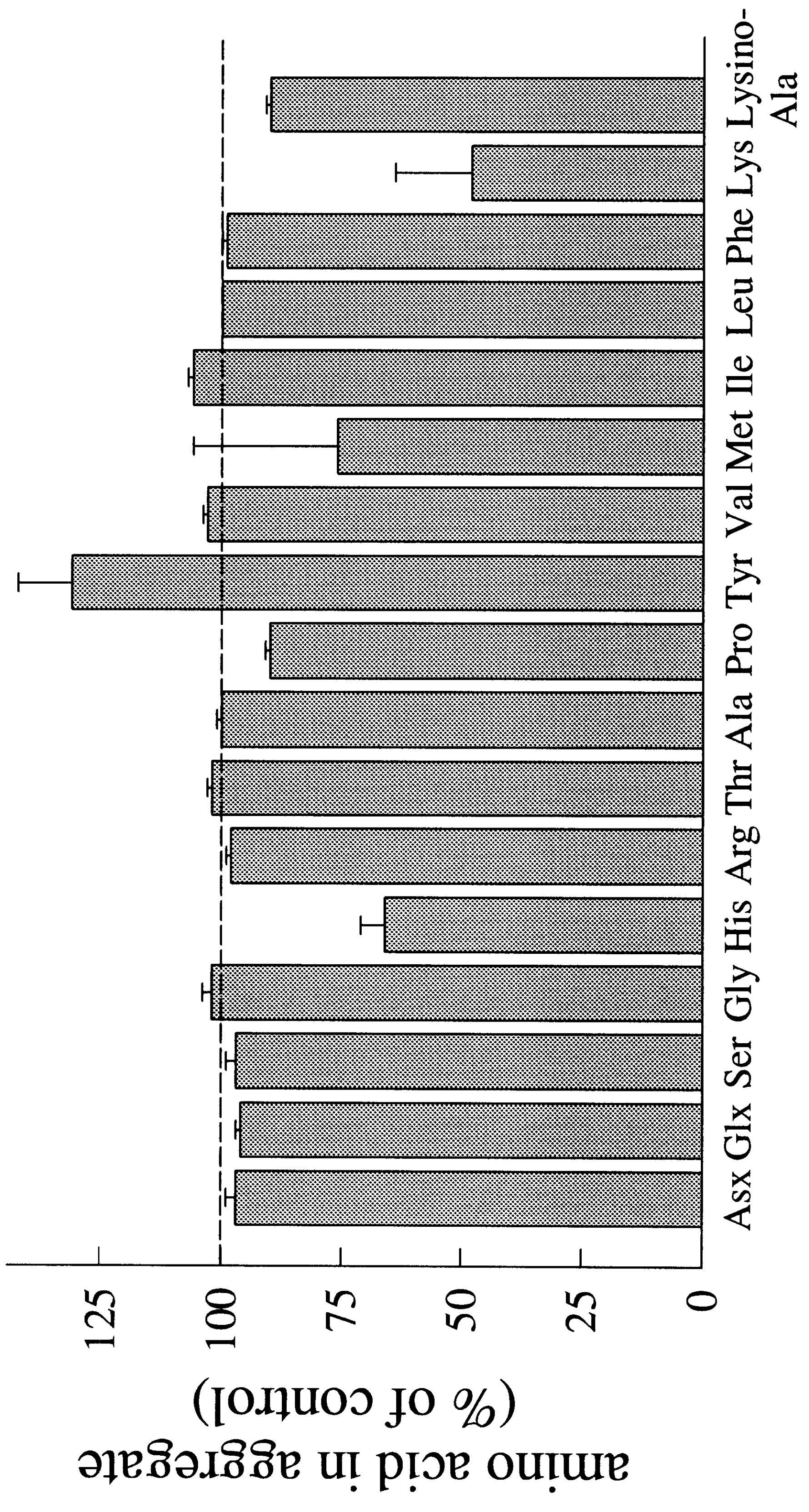
[b] Note that separate calibration curves were obtained for protein determinations in the presence of the additives listed.

[c] PB:sodium phosphate buffer, 100 mM pH 7.3.

[d] Denotes that the amount of aggregates dissolved in these solvents was ≤ amount of unincubated TT dissolved.

[e] DTT:dithiothreitol.

Figure 6.3 Changes in the amino acid composition of TT due to moisture-induced aggregation. Aggregates were obtained following a 9-day incubation at 37°C and 86% relative humidity. The amino acid composition is presented relative to that of unincubated vaccine: Asx, aspartic acid and asparagine; Glx, glutamic acid and glutamine; Ser, serine; Gly, glycine; His, histidine; Arg, arginine; Thr, threonine; Ala, alanine; Pro, proline; Tyr, tyrosine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; Lys, lysine; Lysino-Ala, lysinoalanine. All amino acid concentrations were normalized for the concentration of Leu. The data represent the mean and standard error of two independent measurements.


amino acid in aggregate
(% of control)
0
25
50
75
100
125
Asx
Glx
Ser
Gly
His
Arg
Thr
Ala
Pro
Tyr
Val
Met
Ile
Leu
Phe
Lys
Lysino-
Ala

as determined by the mean data of three experiments, but in this case the standard deviation was so large–30%–that one cannot make any definite conclusions.)

Of these residues, Lys is known to participate in at least two protein cross-linking mechanisms, transamidation (Fig 2.9) and formation of lysinoalanine cross-links (Fig 2.4). The former possibility can be ruled out since it dictates that the amount of Lys measured following acid hydrolysis should be the same for transamidated protein and the control. (Any amide bonds formed via transamidation would have been hydrolyzed under the conditions of our amino acid analysis.) The latter can also be ruled out since there was no increase in the amount of lysinoalanine (which is stable during the amino acid analysis) of the aggregates compared to the control. The observation that this non-native cross-link was present even in the control (at approximately 0.03 mol-equivalents) suggests that its formation probably occurred during the formalinization of TT. To our knowledge, these data are the first direct evidence that lysinoalanine cross-links form during the formaldehyde treatment.

Although the possibilities of transamidation and lysinoalanine formation have been ruled out, the amino acid analysis data (Fig. 6.3) strongly suggest that Lys is involved in TT's moisture-induced aggregation. Lys is known to react with carbonyls, as in the Maillard reaction (Fig. 2.8), for example. Such a group is found in formaldehyde, which is the active agent present during TT's formalinization. Thus, it is logical to further consider this cross-linking agent as a possible participant in TT's moisture-induced aggregation.

6.4.4 The formaldehyde-mediated aggregation hypothesis. Formaldehyde is know to react with Lys, in addition to other amino acid residues such as those of cysteine (Cys), His and Tyr (Means and Feeney, 1971). Three of these residues, namely Lys, His, and Tyr, were quantitatively different in the moisture-induced aggregates vs. the control (Fig. 6.3). Building on these observations, Schwendeman *et al.* (1995c) have hypothesized a framework for TT's moisture-induced aggregation that is mediated by formaldehyde (Figure 6.4).

During formalinization of TT, formaldehyde may react with the residues of Lys or His. In this process, new covalent bonds are formed between the carbonyl carbon of formaldehyde and the ε-amino (side chain) nitrogen atom of Lys or the non-protonated (that is, nucleophilic) nitrogen of the imidazole ring of His. The result is a hydroxymethyl derivative, as depicted in Fig. 6.4 for the case of Lys. In this manner, potentially reactive electrophilic species may be "stored" as labile linkages with TT as a result of formalinization. Another possible mode of storage is within intramolecular aminal-type linkages with another amino group via a Schiff base intermediate (Farrar, 1968; Schwendeman *et al.*, 1995c). This view is consistent with reports of formaldehyde being released during the acid hydrolysis of formalinized proteins (Blass *et al.*, 1967).

Upon condensation, the hydroxymethyl derivative is transformed into a Schiff base imine. This reactive species can conceivably cross-link with another amino group (as discussed above) or with Tyr (Blass *et al.*, 1967; Schwendeman *et al.*, 1995c). If the initial formaldehyde-linked moiety is Lys, the most likely mode of cross-linking is with Tyr (Fig. 6.4). This is because this carbon-carbon linkage is more stable than the carbon-nitrogen one formed between the Schiff base imine and an amino group (Blass *et al.*, 1967; Farrar, 1968; March, 1985). The same applies if the initial formaldehyde-linked moiety is His–the most likely cross-link is with Tyr.

In this manner, formaldehyde can mediate cross-linking in TT between residues of Lys and Tyr (Fig. 6.4) or His and Tyr. This hypothesis does not contradict our solubility data (Table 6.4) which indicates that a covalent, non-disulfide bonded aggregation pathway had occurred. Furthermore, this view is consistent with the loss of Lys and His in the aggregates (Fig. 6.3), as they may be tied up in non-acid hydrolyzable linkages. Moreover, the increase in Tyr observed in the aggregates (Fig. 6.3) may also be explained, as follows. Upon acid hydrolysis of TT, formaldehyde is liberated (Blass *et al.*, 1967) probably from storage in reversible hydroxymethyl or aminal linkages (Schwendeman *et al.*, 1995c). Liberation from the (unincubated) control would be greater than from the aggregates, since in the latter case some formaldehyde had been lost due to its involvement in irreversible, non-acid-hydrolyzable cross-links. In turn, this larger pool of formaldehyde in the control would lead to a lower level of Tyr, since formaldehyde is known to react with phenols (e.g., Tyr) in acidic media (Blass *et al.*, 1967; March, 1985).

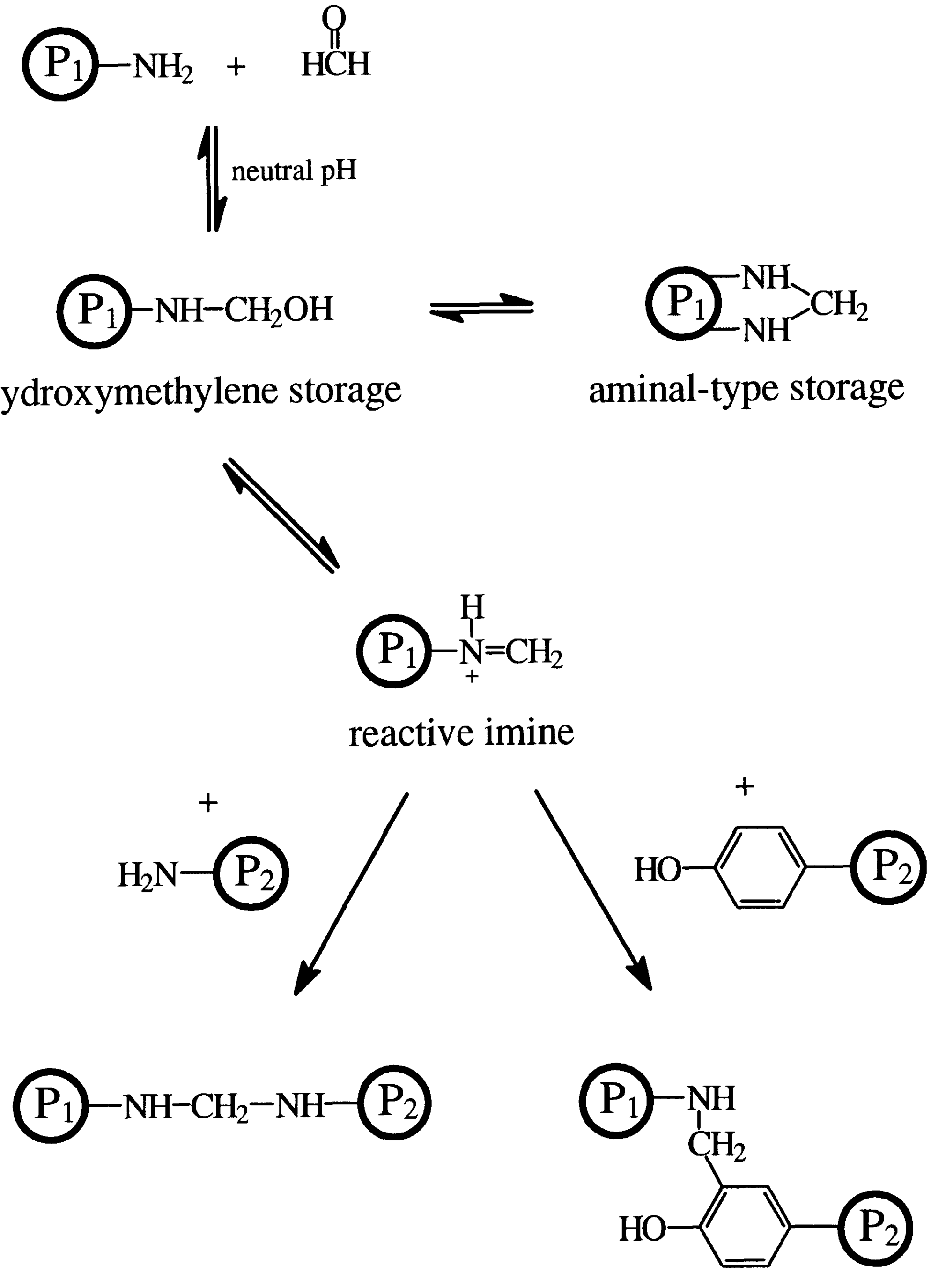


Figure 6.4 A hypothesized pathway for protein aggregation that is mediated by formaldehyde. The reaction of formaldehyde with lysine. The reactive species may be stored reversibly on the protein as either the *N*-hydroxymethyl lysine or the aminal moiety. Upon condensation, a Schiff is formed which can cross-link with either lysine or tyrosine.

6.5 Development of Rational Stabilization Strategies

TT exhibits at least three aggregation pathways in the solid state: non-covalent bonding; covalent, disulfide bonding; and, covalent, non-disulfide bonding hypothesized to be mediated by formaldehyde. Furthermore, the route and extent of aggregation depend on the level of moisture. With such a complex system, it is necessary to take a systematic and rational approach to improve TT stability in the solid form.

6.5.1 Effect of chemical modification on TT solid-phase stability. The two covalent aggregation pathways may be targeted by chemical modification of the reactive groups involved. The formation of disulfide cross-links can occur to TT either by thiol-disulfide exchange (Fig. 2.6) or by β-elimination followed by thiol catalyzed disulfide interchange (Fig. 2.7). Cystines are required for both of these processes. Therefore, reduction of cystines may improve TT's stability in the solid phase. However, we found that reduced TT exhibited a stability comparable to the unmodified TT (Figure 6.5). Note that stability was compared at a humidity of 86%; it was at higher rh (96%) where intermolecular disulfide-bonding was observed.

Although the native structure of tetanus toxin contains free thiols (six) and disulfides (two) (Bizzini *et al.*, 1977; Eisel *et al.*, 1986), the integrity of these moieties following formalinization is uncertain. To investigate this, we used 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Riddles *et al.*, 1983) to measure the accessible free thiols. Virtually no free thiols were found in the unmodified TT. There are two possible explanations for the discrepancy between the data and the value for the native tetanus toxin. The first is that free thiols of Cys were chemically altered during formalinization. A second possibility is that thiols may be buried within the TT molecule, and thus not available to react with DTNB under the non-denaturing conditions used in the study. The reduction with DTT liberated about 1-2 mol-equivalents of thiols. This indicates that only about one cystine was present in formalinized TT. The most likely candidate is the disulfide bond involving Cys^{467} of the H-chain that links the H- and L-chains of TT (Eisel *et al.*, 1986).

A more profitable stabilization strategy is to target the covalent, non-disulfide pathway that was dominant at both intermediate (80%) and high (96%) rh. According to the formaldehyde-mediated aggregation hypothesis, amino groups may be entangled in this pathway either in reversible "storage" of formaldehyde or in irreversible cross-links. Therefore, one strategy to minimize aggregation would be to chemically modify this group, rendering it innocuous. This was accomplished by treatment with succinic anhydride (Hollecker, 1989) prior to lyophilization. In order to determine the efficiency of this procedure, solvent-accessible amino groups were measured using 2,4,6-trinitrobenzenesulfonate (TNBS). This procedure was performed on unmodified, reduced, and reduced and succinylated TT.

The amount of Lys present in TT was determined to be 12 mol-equivalents (Table 6.5). This is strikingly lower than the 107 putative residues in the native tetanus toxin amino acid sequence (Eisel *et al.*, 1986). The same arguments provided earlier to explain the low level of thiols can be invoked here for Lys: either the ε-amino group of Lys becomes chemically modified during the formalinization of TT or these residues are buried within the TT molecule, and thus inaccessible to the DTNB reagent used to measure them. Since the majority of Lys is usually found on a protein's surface (Hollecker, 1989), it seems highly unlikely that such a very large percentage of them would be buried for TT. Therefore, it is more likely that the some 89% loss of Lys had resulted from the formalinization process. This scenario is consistent with the hypothesized aggregation scheme, whereby Lys residues may be converted into their hydroxymethyl- or lysinomethyl derivatives (Fig 6.4A).

Following reduction, the titratable amino groups increased slightly to some 17 mol-equivalents (Table 6.5), probably due to an increased exposure of Lys upon cleavage of the disulfide bond connecting the H- and L-chain of TT. Upon succinylation, the amount of Lys was only 2.6 mol-equivalents. Thus, succinylation was highly successful, reducing the titratable amino groups of TT by about 6-fold. In addition to reacting with Lys, succinic anhydride may also react with thiols and the hydroxyls of serine (Ser), threonine (Thr), and Tyr (Dawson *et al.*, 1986), which may also be involved in the

Table 6.5

Determination of accessible amino groups in TT[a]

Vaccine	Free amino groups[b] (mol-equivalents)
TT	12
Reduced[c]	17
Reduced and succinylated	2.6
CBH-reduced[d]	14
DT	5.2
Succinylated	0.12

[a] All determination were accomplished in non-denaturing media.

[b] Amino groups were determined with 2,4,6-trinitrobenzenesulfonate using α-*N*-acetyl-L-lysine methyl ester as a standard.

[d] Reduced with dithiothreitol.

[d] Reduced with sodium cyanoborohydride (CBH).

hypothesized formaldehyde-mediated aggregation pathway. Moreover, another potential reactant is hydroxymethyl lysine, which is also hypothesized to play a role.

Thus, there is ample reason to expect that the treatment with succinic anhydride should be successful in preventing TT solid-phase aggregation. This was confirmed experimentally. TT which had been succinylated exhibited remarkable stability; essentially no aggregation was observed, even after 9 days of incubation at 37°C and 86% rh (Fig. 6.5). This success is highly consistent with the proposed covalent, non-disulfide-bonded aggregation scheme (Fig 6.4). Therefore, other strategies targeting this pathway should be successful as well.

Another such approach is reduction of the reactive electrophiles, the Schiff base intermediates (Fig. 6.4), to an inactive amine. To test this idea, TT was treated with cyanoborohydride (CBH) and its stability was compared to that of the unmodified vaccine (both samples were lyophilized from 10 mM sodium phosphate, pH 7.3). As illustrated in Figure 6.6, this approach was successful in significantly reducing TT solid-phase aggregation. For example, after a nine-day incubation at 86% rh and 37°C, the CBH-reduced TT aggregated only 41±2%, whereas the unmodified protein aggregated 86±6%.

6.5.2 Effect of co-lyophilized excipients on TT solid-phase stability. Besides chemical modifications, another tactic for stabilizing TT is addition of excipients which are capable of stabilizing TT during solid-state incubation. For example, TT aggregation may be affected by controlling the level of moisture on the protein (Section 6.3.1). Specifically, at very high levels of moisture (>50 g water/100 g protein), TT solid-phase aggregation markedly decreased (Fig. 6.2A). Therefore, it was of interest to test excipients of varying water-sorbing abilities for their stabilizing potencies. We selected excipients representing a range of water-sorbing powers (Table 6.6): high, NaCl (349 g water/100 g sorbed at 86% rh and 37°C); medium, sorbitol (47 g water/100 g); and, low, PEG (M_w=20 kDa) (5.3 g water/100 g). These were then co-lyophilized with TT, all at 1:5 excipient:protein (g/g). The aggregation of these samples, compared to that of TT lyophilized without excipients, is shown in Figure 6.7.

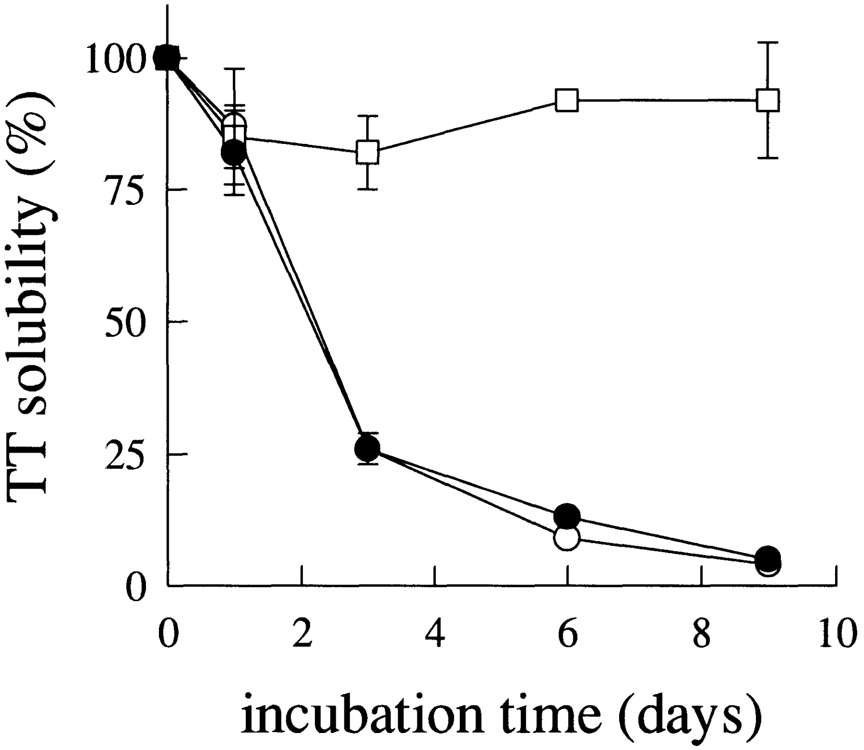


Figure 6.5 Stabilization of TT against moisture-induced aggregation afforded by succinylation. The time-course of solubility at 37°C and 86% relative humidity is depicted for lyophilized samples of: [●], unmodified TT; [○] reduced TT; and [□] TT that was reduced, and succinylated.

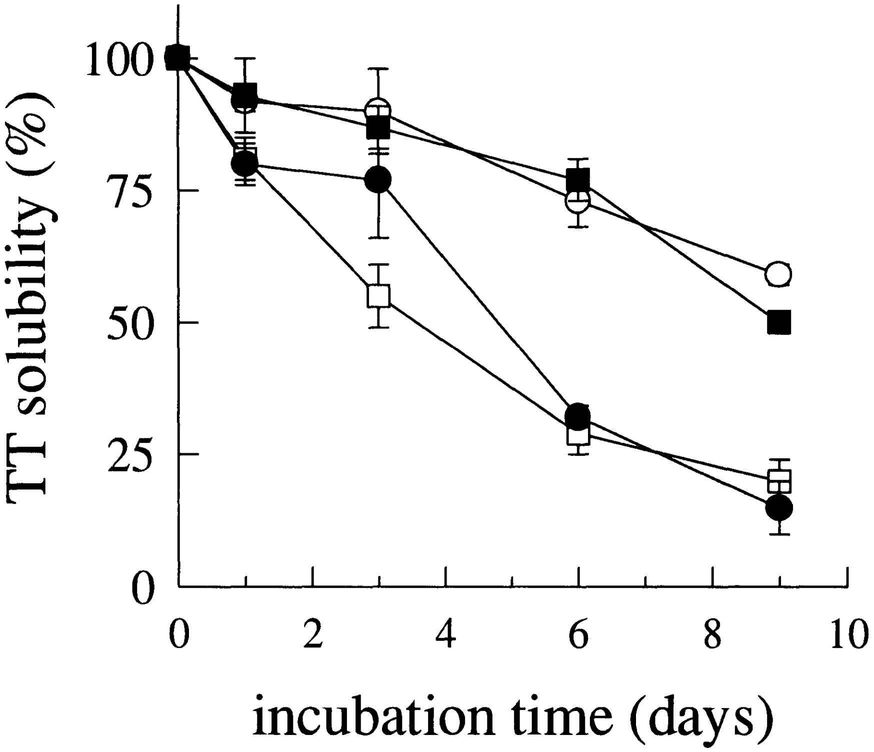


Figure 6.6 Stabilization of TT against aggregation afforded by treatment with cyanoborohydride. The time course of solubility at 37°C and 86% relative humidity is shown for: [●] unmodified TT; [○] cyanoborohydride-reduced TT; and TT co-lyophilized (1:5 g/g) with [■] glycine and [□] α-*N*-acetyl-L-lysine methyl ester. These samples were all lyophilized from 10 mM sodium phosphate, pH 7.3. Incubation conditions are the same as in Fig. 6.5.

Table 6.6

Water sorption of various excipients

Compound[a]	Water content, wetted[b] (g/100 g dry solid)
sodium chloride	349±6
sodium phosphate[c]	59±8
sorbitol	47±2
α-*N*-acetyl-L-lysine methyl ester	46±3
glycine	7±4
poly[ethylene glycol] (PEG)	5±1

[a] All compounds were initially lyophilized from 1 mg/ml, pH 7.3.

[b] Water content was measured by Karl Fisher titration following a 2-week incubation at 37°C and 86% rh.

[c] Mixture of mono- and dibasic salts.

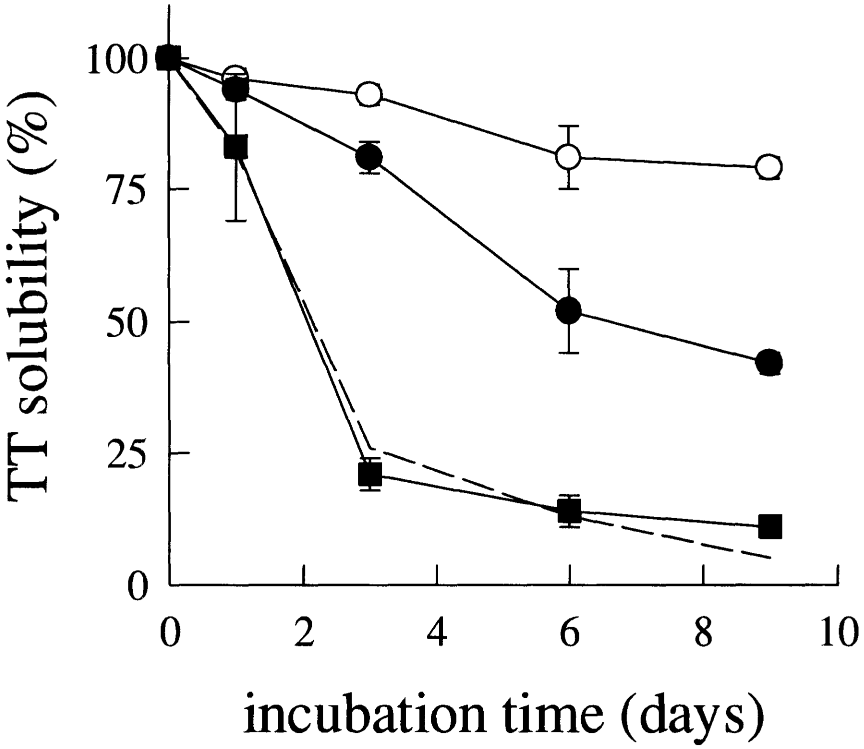


Figure 6.7 Stability of TT lyophilized with excipients of varying water-sorbing potencies. Data were obtained for TT co-lyophilized with 1 g:5 g dry protein of : [○] sorbitol; [●] NaCl; and [■] poly[ethylene glycol]. Data for TT in the absence of excipient (from Fig. 6.5) are the dashed curve. Incubation conditions are the same as in Fig. 6.5.

As predicted, the addition of NaCl (partially) inhibited TT solid-phase aggregation, while PEG was totally ineffective, as compared to TT that was freeze dried without excipients. In contrast, the presence of sorbitol, which should not alter appreciably the water content in, and thus, the stability of, the vaccine powder had a remarkable stabilizing effect. This indicates that water-sorbing capability is not the only important characteristic of the excipient that influences solid-state aggregation.

It is important to note that all TT samples from which the data were obtained in Fig. 6.7 (including the one without excipients) were lyophilized from an aqueous solution containing 1 mM sodium phosphate, roughly corresponding to 1 g:7 g protein. This compound had a higher water-sorbing capability (59±8 g/100g; Table 6.6) than the standard TT lyophilized formulation (36±1 g/100 g for TT which was lyophilized from 1 mM sodium phosphate). Thus, it is noteworthy that when lyophilized from a 10-fold higher level of sodium phosphate, 10 mM, corresponding to approximately 1.4 g:1 g protein, TT stability was slightly higher (compare the data for filled circles in Figs. 6.5 and 6.6). This indicates that a strongly water-sorbing excipient (for example, NaCl) is required to see a major stabilization effect. This view is similar to the effect observed for the stabilization of rHA with strongly water-sorbing excipients (Section 4.6.1).

Besides strongly water-sorbing compounds, another useful class of excipients would be those that compete with protein reactive groups for the aggregation reaction. Instead of deleterious protein-protein reactions, there would simply be reaction of protein with the excipient. To explore this idea, TT was co-lyophilized (1 g:5 g protein) with two excipients, glycine and α-*N*-acetyl-L-lysine methyl ester, which contain amino groups that could compete with Lys on the protein, as dictated by the proposed aggregation scheme (Fig. 6.4).

The stabilities of these formulations, as compared to TT lyophilized alone (all samples were lyophilized from 10 mM sodium phosphate) are depicted in Fig. 6.6. For the case of TT co-lyophilized with glycine, there was indeed some improvement in stability. After a nine-day incubation at 86% rh and 37°C, the extent of aggregation was 50±2% in its presence of glycine compared to 86±6 aggregation in its absence. Note that this improvement cannot be the result of increased hydration of the lyophilized powder, since glycine exhibited very low water sorption under the conditions used (Table 6.6). Aggregation was not completely ameliorated, however, perhaps due to glycine's inability to affect cross-linking via non-covalent or disulfide bonds. This view is consistent with a report demonstrating that TT solid-phase aggregation at 50°C and 96% rh occurs by these two pathways (and not covalent, non-disulfide bonding) where the amino acid, glutamate, was added as a stabilizer (Schwendeman *et al.*, 1995d).

On the other hand, the addition of α-*N*-acetyl-L-lysine methyl ester did not significantly effect TT's moisture-induced aggregation (Fig 6.6). Since the amino group of this compound has a pK_a higher than that found in amino acids (Means and Feeny, 1971), it is less nucleophilic than glycine, which may explain its ineffectiveness. In addition, since it has a significantly higher molecular weight than glycine, there are fewer mol-equivalents of amino groups present than in the glycine case. Note that α-*N*-acetyl-L-lysine methyl ester had a similar affinity for water as did sorbitol (Table 6.6), a further indication of the latter's distinct mode of stabilization. The data demonstrate that using excipients as competitors will, at best, only result in partial stabilization. The other approaches developed for stabilization of TT in the solid phase, namely chemical modification of reactive groups, and addition of strongly water-sorbing excipients, are favored.

6.6 Lyophilization-Induced Structural Rearrangement of TT and the Effect of Excipients

Although the use of strongly water-sorbing excipients as stabilizers for solid TT is recommended, the data indicate that capacity to bind water is not the only factor dictating potency. If so, sodium chloride should have been more effective than sorbitol in this regard. In fact, the opposite trend was observed (Fig. 6.7). Since it was shown that TT undergoes structural rearrangement upon lyophilization (Section 6.2) and that certain excipients may guard against this process in other proteins (Arakawa *et al.*, 1991, Prestrelski *et al.* 1993a, 1993b; Desai *et al.*, 1994; Costantino *et al.*, 1995b; Griebenow and Klibanov, 1995), we investigated the relationship between the structure and stability of solid TT in the presence of excipients.

To this end, FTIR was used to probe the secondary structure of TT in the various excipient:TT formulations for which data were presented in Fig 6.7. In each of the three cases studied (TT co-lyophilized with NaCl, sorbitol, and PEG at 1:5 g:g protein) we found that there was perturbation of TT's secondary structure upon lyophilization (Table 6.2). For example, in each case, the α-helix content was lower than that in aqueous solution.

Among the minor differences observed, it was revealed that when co-lyophilized with sodium chloride (which imparted partial stabilization; Fig. 6.7), there was a slightly more pronounced α-helix to β-sheet conversion upon lyophilization compared to TT lyophilized alone (Table 6.2). Sorbitol (a very effective stabilizer; Fig. 6.7) had little effect on the structure of lyophilized TT (Table 6.2). Although others have shown that sorbitol may help to retain a more solution-like structure upon lyophilization (Prestrelski *et al.*, 1993a; Desai *et al.*, 1994; Griebenow and Klibanov, 1995), at least 1:1 g sorbitol:g protein was required to see the effect. The sorbitol loading herein, namely 1:5, was far lower. Another observation of some note was that presence of PEG (no effect on aggregation) during lyophilization resulted in slightly less β-sheet formation and slightly more unordered structure.

Thus, there was no clear relationship between solid TT's secondary structure and its stability in the presence of excipients. Furthermore, the data presented earlier (Fig. 6.6) indicated that water-sorbing capacity is not the only factor dictating an excipient's stabilizing potency. Therefore, other (environmental) factors must also influence TT's stability. A parallel conclusion was drawn regarding the stability and secondary structure of rHA.

6.7 Concluding Remarks

Herein, we report that lyophilized tetanus toxoid, a protein vaccine, aggregates upon exposure to 37°C and elevated humidity. The mechanism and extent of aggregation was dependent on the moisture level, pointing to the critical role of protein conformational mobility. The dominant pathway, heretofore undescribed, was covalent, non-disulfide bonding. We hypothesize that this process is mediated by formaldehyde (present during the formaldehyde treatment, or formalinization, of the vaccine) and ultimately results in various cross-links involving lysine, histidine, and tyrosine. Various rational stabilization strategies were proposed and tested. Those highly successful include succinylation of ε-amino groups of lysine, treatment with cyanoborohydride to reduce Schiff bases (which are intermediates in the aggregation process), and addition of certain excipients.

Our findings suggest that the stability of formalinized proteins will be an important consideration in their development as sustained-release vaccines. In addition, since we have specifically targeted the proposed formaldehyde-mediated aggregation pathway, our stabilization strategies may be applicable to other formaldehyde-treated vaccines as well. To test this, we chose another, very different formalinized vaccine, DT, and found that it too was susceptible to moisture-induced aggregation at 37°C and elevated (86%) relative humidity (Figure 6.8). Furthermore, we also succinylated the protein, with a high degree of efficiency (Table 6.5) and found, as predicted, a dramatic increase in its stability (Fig. 6.8).

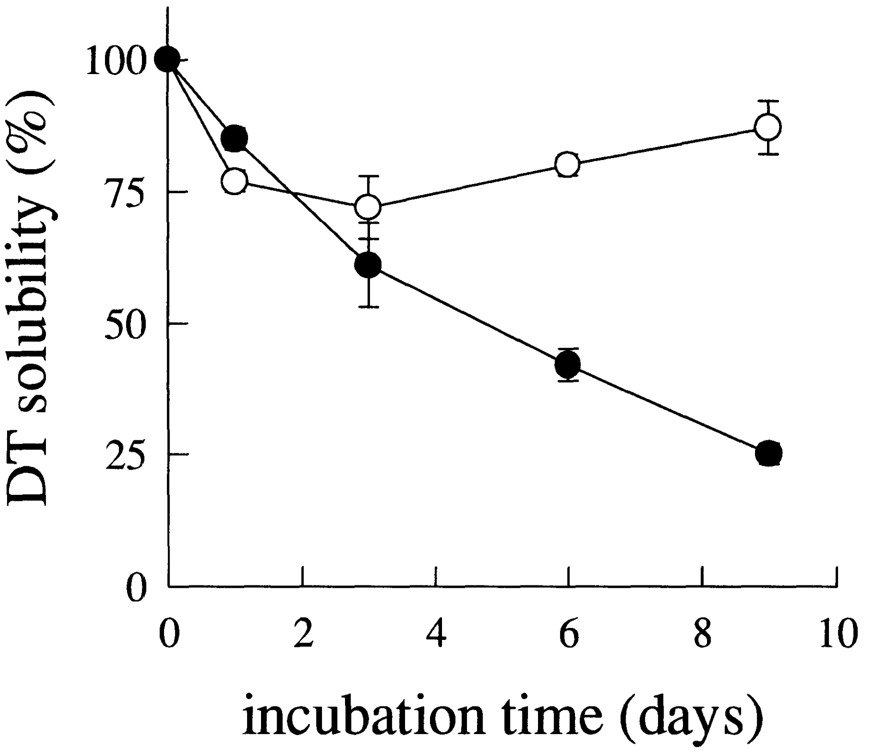


Figure 6.8 Solid-phase aggregation of DT and stabilization afforded by succinylation. The time-course of solubility at 37°C and 86% relative humidity is depicted for lyophilized samples of [●] unmodified DT and [○] succinylated DT. These samples were lyophilized from 10 mM sodium phosphate, pH 7.3.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

In order to successfully employ proteins as pharmaceuticals, it is essential to understand mechanistically the stability issues involved in their formulation and delivery. Various deleterious processes may occur in protein formulations, thereby diminishing their therapeutic value. This thesis has focused on one aspect of this problem, namely aggregation of solid proteins under pharmaceutically relevant conditions (elevated temperature and water activity). Strategies to pursue such studies have been elaborated with an emphasis on a mechanistic analysis of aggregate formation.

We have investigated the solid-state stability of three pharmaceutical proteins and discovered that they are susceptible to aggregation in the presence of moisture which depends on the protein and its environment. Both covalent and non-covalent aggregation pathways have been uncovered. A protein that contains disulfide bonds as well as free thiols (recombinant human albumin (rHA)) aggregates via thiol-disulfide interchange. For a protein which contains disulfides but not free thiols (insulin), intermolecular disulfide bonding may still occur when disulfides undergo β-elimination yielding free thiols which can catalyze disulfide scrambling. Finally, a protein containing no cysteine/cystine residues may aggregate by other covalent pathways or by non-covalent routes. A protein that has been formalinized (specifically, a vaccine, such as tetanus toxoid (TT), that has been detoxified by treatment with formaldehyde) may be susceptible to formaldehyde-mediated cross-linking involving lysine, histidine, and tyrosine residues.

In the case of solid-state thiol-disulfide exchange, stabilization was achieved by lowering the pH prior to lyophilization. This results in protonation of free thiols, thus rendering them incapable of involvement in the thiol-disulfide exchange reaction. This approach was also successful in arresting the pathway of β-elimination, followed by thiol-catalyzed disulfide interchange, since hydroxide ion is necessary to carry out the β-elimination step. Another successful approach in this case was to oxidize free thiols by the presence of transition metal cations (most notably Cu^{2+}) added prior to lyophilization. For the instance of the formalinized vaccine, TT, solid-state stabilization was achieved by blocking reactive amino groups by succinylation and by reduction of a critical reactive intermediate species (hypothesized to be a Shiff base imine) with cyanoborohydride, both prior to lyophilization. These examples demonstrate how mechanistic study of protein aggregation in the solid phase may lead to the rational design of more stable and effective pharmaceutical protein formulations.

What other possible pathways lead to protein aggregation in the solid state, and can one employ the same rational approach towards combating such processes? This is clearly an area for future research. For example, it has been proposed that lyophilized somatotropin (Hageman, 1988; Hageman *et al.*, 1992a) and ribonuclease A (Townsend and DeLuca, 1988, 1991) form intermolecular cross-links (during their storage) via the transamidation reaction. It remains to be seen how commonly this mechanism occurs in solid proteins.

Another area for future work is the application of the general approach elaborated herein towards improving stability against intramolecular processes occurring in solid proteins. For intramolecular cross-linking involving disulfides or lysines, one can invoke strategies discussed above for the intermolecular case. If, for example, hydrolysis occurs due to a low pH, one can adjust it to a higher value by either lyophilizing from a more basic pH or by addition of buffering agents to the formulation. Since deamidation is favored at high pH, in this case a similar strategy can be invoked to lower the pH.

However, before such stabilization studies can be conducted, it is first necessary to uncover which intramolecular processes are exhibited by proteins during solid-state incubation. Reports of these are scarce. Model peptides (Oliyai *et al.*, 1994) and at least one protein, somatotropin (Hageman, 1992a), undergo deamidation in the solid phase. Incubation of somatotropin also leads to oxidation of cysteines (Pearlman and Nguyen, 1992). How common are such events in various proteins and what other intramolecular pathways may occur? This is an important area for future investigation.

A general approach to improve the stability of pharmaceutical proteins may be to control the physical, or conformational, stability. It is now clear that for solid proteins there is a significant structural rearrangement upon dehydration (Prestrelski *et al.*, 1993b; Desai *et al.*, 1994; Griebenow and Klibanov, 1995; Costantino *et al.*, 1995b). This loss in native structure may result in aggregation via hydrophobic interactions or higher rates of other deleterious mechanisms if there is an increased exposure of reactive groups (Manning *et al.*, 1989; Volkin and Klibanov, 1989; Cleland *et al.*, 1993). Herein, it was demonstrated that rHA and tetanus toxoid experienced significant (largely reversible) rearrangements of secondary structure upon lyophilization, as determined by Fourier-transform infrared spectroscopic analysis. In both cases, there was a significant loss of α-helices with a concomitant increase in β-sheets. For rHA, there was also a marked increase in unordered structures, suggesting a partial unfolding. Although such events may be a prerequisite for solid-phase aggregation, other, environmental factors strongly influence stability.

Yet another general approach to stabilizing solid proteins, against either deleterious inter- or intramolecular reactions is to control the amount of water in vicinity of the protein at optimal levels. As a rule, the lower the water content, the higher the stability. For example, we found that the moisture-induced aggregations of rHA, insulin, and TT were all critically dependent upon the level of moisture on the protein. All three proteins were essentially stable if maintained at moisture levels below their monolayer water sorption. As the level of moisture was raised, solid-state aggregation ensued as a result of increased protein flexibility. In all but the case of insulin, the dependency of aggregation on moisture was bell-shaped. Therefore, to avoid deleterious processes, it is necessary to avoid that particular water content which leads to instability. As a consequence, we found that strongly water-sorbing excipients are quite potent stabilizers, as exemplified by our investigation of many different excipients on the stability of rHA. It is important to note that as a protein becomes increasingly hydrated (as in a polymeric matrix) and passes through a deleterious water content, both the kinetics of water sorption and aggregation play a role; it is desirable to minimize the time that a protein spends at a particularly deleterious water content. A closer look at the relationship between kinetics of aggregation and kinetics of water sorption for solid proteins is yet another interesting avenue for further study.

Why are freeze-dried rHA and TT more stable at high moisture levels (that is, >50 g water /100 g rHA and >100 g water/g TT) than at intermediate ones? One factor is that by increasing the water, molecular contacts are diminished, diluting the protein, which decreases intermolecular processes. In principle, this effect can be achieved not only by addition of water but by addition of excipients. Specifically, we have shown above that this rationale explains why the addition of dextran to rHA (both have very similar affinities for water) imparts extra stability.

We have reported, for the first time, another important effect, i.e., that upon increasing the water content a protein (as exemplified with rHA) can refold back into its native and more stable conformation. This view is consistent with the strong dependence between rHA's stability and its water content. Whether this effect is important in stabilization of other solid proteins against moisture-induced aggregation is an intriguing area for future study.

With the recent developments in FTIR spectroscopy, it is now feasible to address this issue. For example, is dehydration-induced structural rearrangement in secondary structure (as determined by inspection of the vibrational spectrum in the amide III region) a prerequisite for aggregation during sold-phase incubation? Will one observe solid-phase aggregation under conditions where the structure has somehow been preserved? The data herein, which demonstrate that the *extent* of the process does not appear to correlate with solid-phase instability, do not directly address this question. Another, related question is: what is the structure of moisture-induced aggregates compared to the native and dehydration-perturbed protein? Although this question has been addressed for aggregation of proteins in solution (Kato and Takagi, 1988; Görne-Tschelnokow *et al.*, 1993; Kim *et al.*, 1994), there are no reports of this kind regarding aggregation induced by incubation of solid protein at elevated temperature and moisture.

Other, related questions may also be probed using FTIR spectroscopy. For instance, at what point during rehydration does a lyophilized protein with a perturbed secondary structure refold to its native form? Toward this goal, one can examine the secondary structure of lyophilized protein stored at various humidities (and thus having different water contents). Yet another area where little work has been conducted is the structure of solid protein suspended within polymeric matrices, and the relationship to stability within the depot. In order to address this issue with FTIR spectroscopy, it will be necessary to overcome any potential overlapping in the vibrational spectrum due to the presence of both protein and polymer.

An additional area of current and future research is investigation of the relationship between stability and T_g, the glass transition temperature of the solid protein formulation. It is common wisdom that, in general, reactivities are higher (and thus stabilities lower) in the rubbery state (above T_g) than in the glassy one (Franks *et al.*, 1991; Franks, 1994; Angell, 1995). Since T_g is highly impacted by factors such as water content and the presence of excipients (Ahlneck and Zografi, 1990; Pikal, 1990b; Franks *et al.*, 1991; Franks, 1994; Hageman, 1992), it would be beneficial to fully understand the effect of T_g upon rates of deleterious inter- and/or intramolecular processes occurring in solid proteins. For example, is the increase in solid-state aggregation with increasing moisture content (the ascending portion of the bell-shaped curve commonly observed for the aggregation dependence) consistent with the lowering of T_g? Specifically, does the maximum for aggregation correspond to the point at which the water content is high enough to result in a T_g lower than the incubation temperature, resulting in "collapse" of the lyophilizate into the rubbery state? Since it is observed that further increase in water content often stabilizes proteins (the descending portion of the aggregation dependency), it is clear that T_g is only one of the factors influencing protein solid-state stability. In a recent model study (solid-state degradation of aspartame), it was shown that it is the water activity and not the T_g which dictates solid-state reactivity (Bell and Hageman, 1994). More work is necessary to clarify the controversial roles of the glass transition phenomena and water activity on the rates of deleterious (protein) reactions.

Yet another, important area for future consideration is extension of the knowledge gained from mechanistic studies of solid protein deterioration in stabilizing solid proteins within actual pharmaceutical formulations *in vitro*, and ultimately, *in vivo*. One such investigation, the evaluation of succinylation in improving the release of TT from poly[lactic-*co*-glycolic acid] microspheres, is currently underway (Schwendeman *et al.*, 1995d).

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APPENDIX A

ON THE IONIZATION STATE OF COMPOUNDS IN THE SOLID AND AQUEOUS FORMS

A.1 Motivation and Overview

Lyophilization from acidic pH resulted in formulations of rHA (Section 4.9) and insulin (Sections 5.2.4 and 5.4.1) that exhibited superior stabilities towards moisture-induced aggregation. In the former case, it was hypothesized that the stabilization was due to increased protonation of reactive thiols. (It is the thiolate ion which is the reactive species in the thiol-disulfide interchange; Section 2.2.7). Of course, this hypothesis assumes that the ionization state of thiols in the solid state is the same as that in aqueous solution prior to lyophilization. Heretofore, this issue was not directly addressed.

Previous studies provide indirect evidence that the ionization state of proteins is "remembered" upon lyophilization. For example, it has been shown for freeze-dried proteins placed in organic solvents that properties such as enzymatic activity (Zaks and Klibanov, 1985, 1988; Klibanov, 1989) and solubility (Chin *et al.*, 1994) are markedly dependent upon the pH prior to lyophilization. Specifically, enzyme powders obtained by lyophilization from a pH corresponding to optimal activity in aqueous solution also are maximally active in organic solvents (Zaks and Klibanov, 1988). This effect may be explained by titration of catalytically important active center residues, suggesting a similar ionization state in aqueous solution and in the lyophilized powder (that is, provided that enzymatic activity for solid proteins in organic solvents depends on the ionization state as it does in water (Fersht, 1985)).

Few studies have directly attempted to measure the ionization state of a protein (or any other ionizable organic compound for that matter) in the solid state as a function of that previously in aqueous solution. This is largely due to insufficient methodologies applicable for both liquids and solids. In one study (Munowitz *et al.*, 1982), which employed ^{15}N NMR spectroscopy, it was shown that the midpoint for the transition between cationic and neutral forms of the imidizole ring of histidine (the pK_a) occurred in the lyophilized powder at a "pH" of 6.3 (the pH measured in the aqueous solution prior to lyophilization), slightly higher than that reported in solution (pK_a =6.04; Dawson *et al.*, 1986). Similar ^{15}N NMR investigation of the His^{57} residue in α-lytic protease found that its "pK_a" in the lyophilized powder was 7.0±1.0 (Huang *et al.*, 1984). Although this is within the range observed for the pK_a in aqueous solution (Tanford, 1962), the uncertainty in the measurement does not rule out the possibility of a small change in the ionization state upon lyophilization.

To explore this issue, herein we employed Fourier-transform infrared (FTIR) spectroscopy (a technique which was employed successfully for both liquid and solid proteins; Section 3.3.17) to precisely quantitate the relative concentrations of ionized and neutral species in both aqueous and lyophilized states. This was accomplished by curve-fitting of the spectra in a region where both ionized and neutral species exhibited distinct absorption bands. Since polypeptides exhibit complex spectra with numerous overlapping bands, it was necessary to investigate more simple model compounds having the same ionizable groups found in proteins. Thus, we chose to study the ionizable groups of three model compounds: the carboxyl group of glycine (Gly), the amino group of tris(hydroxymethyl)aminomethane (Tris), and the enol of 3-hydroxybenzoic acid (HBA).

A.2 Using Infrared Spectroscopy to Investigate Ionization State

The Lambert-Beer Law can be used to describe the relationship between IR absorbance (at a given wavenumber) and concentration (Harris, 1987):

$$I=\varepsilon\, C\, l$$

where ε is the extinction coefficient, C is the concentration, and l is the path length. Using this relation, the concentration can also be described by integration over the entire absorption peak,

$$C=\int\frac{I}{\varepsilon\, l}$$

Note that for a given vibrational mode giving rise to an absorption peak, the extinction coefficient (and, of course, the integration range) may change when a group directly influencing the absorption becomes ionized.

Thus, for familiar acid-base equilibria,

$$HA \leftrightarrow A^- + H^+$$

the fraction of ionized species, F, can be expressed in terms of IR absorbance as:

$$F = \frac{[A^-]}{[A^-]+[HA]} = \frac{\int \frac{I_{A^-}}{\varepsilon_{A^-} l}}{\int \frac{I_{A^-}}{\varepsilon_{A^-} l} + \int \frac{I_{HA}}{\varepsilon_{HA} l}} = \frac{1}{1 + \int \frac{I_{HA}\varepsilon_{A^-}}{I_{A^-}\varepsilon_{HA}}} = \frac{1}{1 + \frac{A_{HA}}{A_{A^-}}\left(\int \frac{\varepsilon_{A^-}}{\varepsilon_{HA}}\right)}$$

where the integration is performed over defined IR peaks arising from species HA and A^- yielding the area A_{HA} and A_A^-, respectively. These areas can be obtained from Gaussian curve-fitting of IR spectra (Section 3.3.17). Since the ratio for of the peak areas is determined from the curve-fit of the spectrum, all that is required to calculate F is the integral term involving the ratio of extinction coefficients. It can either be assumed to be unity (if deprotonation only changes the peak wavenumber for a given vibrational mode, and not the extinction coefficient) or it can be determined from the spectrum obtained in aqueous solution at the pH corresponding to the known pK_a value, since at these conditions

$$F = 0.5 \qquad \text{and thus} \qquad \int \frac{\varepsilon_{A^-}}{\varepsilon_{HA}} = \frac{A_{A^-}}{A_{HA}} .$$

Provided that the vibrational peaks have been properly identified, and having established the ratio of extinction coefficients for protonated and deprotonated species, one can determine F for samples at various pHs and, from these data, experimentally calculate the pK_a since

$$F = \frac{1}{1 + \frac{[H^+]}{K_a}} = \frac{1}{1 + \frac{\log^{-1}(-pH)}{\log^{-1}(-pK_a)}} .$$

For both aqueous and solid samples, this experimentally determined pK_a has the usual definition as the midpoint for the transition between ionic and neutral species.

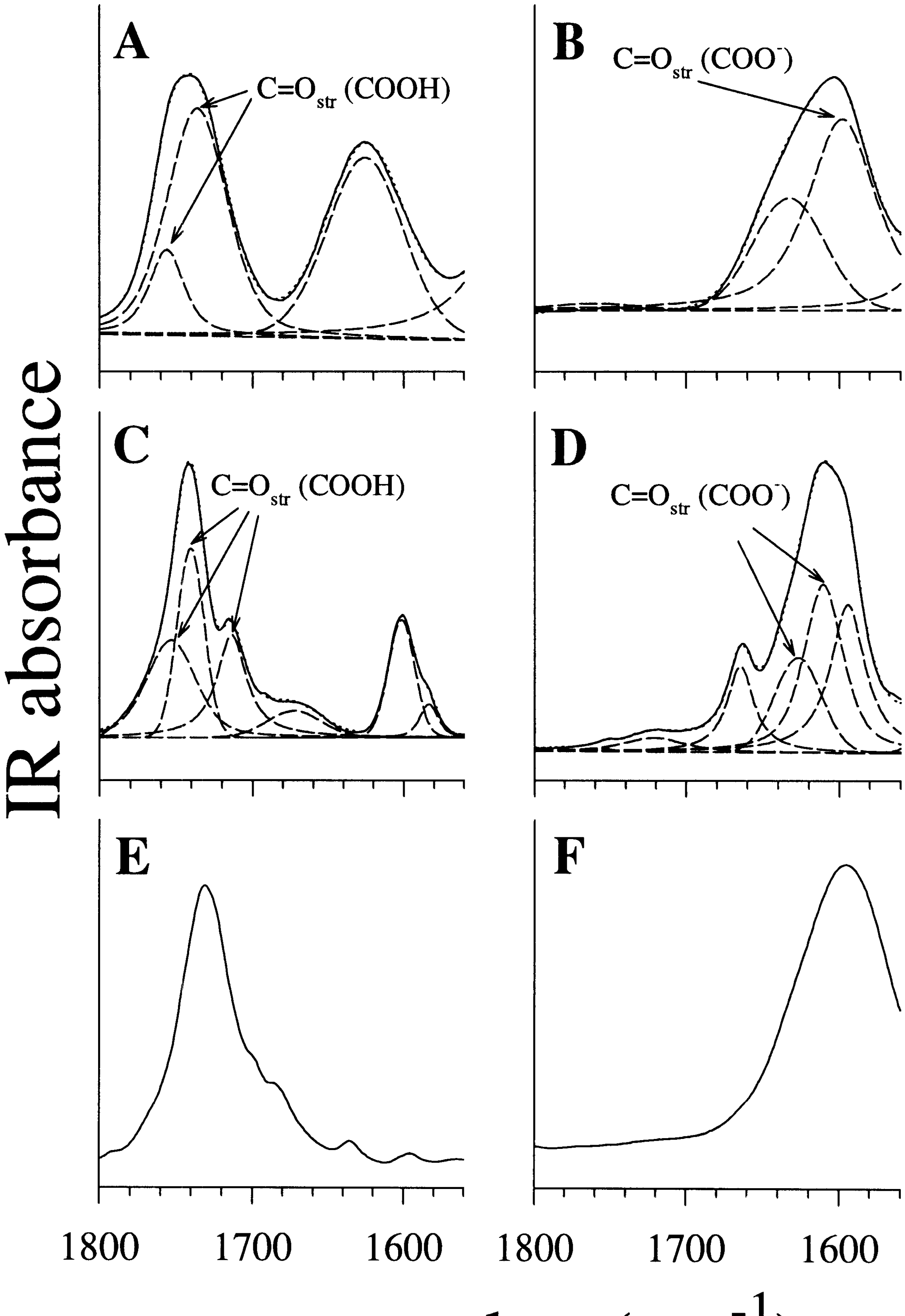
A.3 Study of Model Organic Compounds

Due to the great complexity found in polypeptide spectra, it was necessary to choose simpler organic compounds (containing the same ionizable groups found in proteins) for our study. Such a model compound is the simple amino acid Gly which contains two ionizable groups, carboxyl and amino, commonly found in proteins. Thus, we initially chose to study the effect of dehydration on the ionization state of the carboxyl group of Gly. Since the $C{=}O_{str}$ (COOH) and $C{=}O_{str}$ (COO^-) absorption band maxima for amino acids in water are known to occur at approximately 1740 and 1600 cm^{-1}(Venyaminov and Kalnin, 1990), respectively, we monitored this spectral region.

It was important to first validate our approach by using IR spectroscopy to experimentally determine the pK_a in aqueous solution and compare it with the established literature value. To this end, spectra were obtained for Gly in water at pH ranging from 1.0 to 4.0. At pH 1.0, where the carboxyl group is fully protonated, peaks were present with maxima at wavenumbers (in cm^{-1}) of 1755±1, 1735±1, and 1626±1 (Figure A.1A). The first two peaks were assigned to C=O stretching (of COOH), and the third peak corresponds to an asymmetrical deformation vibration of $-NH_3^+$ groups ($NH_3^+{}_{def,as}$) (Venyaminov and Kalnin, 1990). The vibrational spectrum for Gly in solution at pH 4.0 (Fig A.1B) shows that the $C{=}O_{str}$ (COOH) peaks were virtually absent. Concomitantly, a new band appeared at 1598±1 cm^{-1}, corresponding to $C{=}O_{str}$ (COO^-) (Venyaminov and Kalnin, 1990). Note that the $NH_3^+{}_{def,as}$ vibrational bands were shifted somewhat to 1632±1 cm^{-1}.

Having now assigned all appropriate bands for over the spectral region and pH range of interest, it was possible to quantitate the fraction of ionized species as a function of pH for aqueous Gly. For this analysis, it was presumed that removal of the proton from the carboxyl group did not change the extinction coefficient of the $C{=}O_{str}$ absorption band (that is, it was assumed that $\varepsilon_{0,COO^-}/\varepsilon_{0,COOH}=1$). The plot of the fraction of ionized species (F) as a function of the pH (from 1.0 to 4.0) is shown in Figure A.2A. Curve fitting of these data yielded an experimental value for the solution pK_a of the Gly carboxyl group of 2.29±0.02. This is in excellent agreement with the known value of 2.35 at room

Figure A.1 IR absorption of Gly for various liquid and solid samples. (A) In H_2O, pH 1.0. (B) In H_2O, pH 4.0. (C) Powder, lyophilized from water pH 1.0. (D) Powder, lyophilized from water pH 4.0. (E) In D_2O, pD 1.1. (F) Powder, lyophilized from D_2O pD 4.6. Sample concentrations were 100 mg/ml for liquids and 0.5-2 mg per 200 mg KBr for solids.


A
C=O_str (COOH)
B
C=O_str (COO⁻)
C
C=O_str (COOH)
D
C=O_str (COO⁻)
E
F
IR absorbance
1800
1700
1600
1800
1700
1600
wavenumber (cm⁻¹)

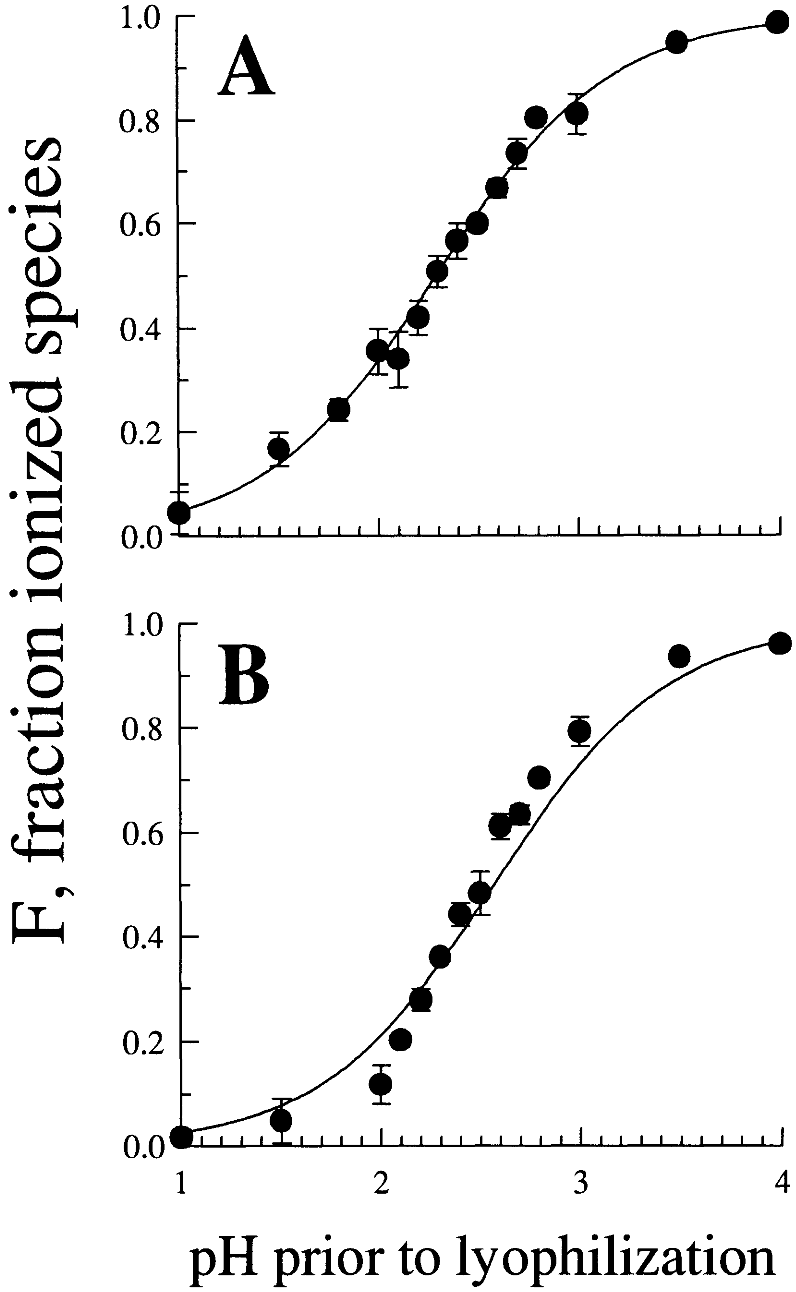


Figure A.2 Determination of midpoint for the ionization of the carboxyl group of Gly in the liquid and solid states. (A) The fraction of ionized species in aqueous solution as a function of the pH. The solid line represents the results of the curve fit: pK_a=2.29±0.02. (B) The fraction of ionized species in the solid state as a function of the pH prior to lyophilization. The solid line represents the results of the curve fit: pK_a = 2.57±0.04. For both liquid and solid determinations, methodologies are discussed in the text.

temperature (Dawson *et al.*, 1986). Thus, our assumption regarding the ratio of extinction coefficients for $C{=}O_{str}$ and, more importantly, our approach in general were validated.

The next step was to use this same approach to determine the fraction of ionized species in the lyophilized state as a function of pH (which, of course, was measured previously in aqueous solution). Consequently, spectra were obtained for solid Gly powders obtained by lyophilization from pHs from 1.0 to 4.0. Several significant changes appeared in the vibrational spectrum as a result of lyophilization. For example, compare the spectra obtained at pH 1.0 for the aqueous solution (Fig A.1A) and the lyophilized powder (Figure A.1C). (In both cases, the carboxyl group should be essentially completely protonated.) First, the $C{=}O_{str}$ (COOH) absorption was more complex for the lyophilized powder, with an additional peak appearing at 1713±2 cm^{-1} (Fig. A.1C). Second, a minor IR absorption band was observed in the region around 1660 cm^{-1}, possibly from the presence of bound water in the solid sample. Third, the IR absorption for $NH_3{}^+{}_{def,as}$ comprised two peaks shifted some 30 cm^{-1} lower (to wavenumbers of 1601±2 and 1583±2 cm^{-1}).

There were also significant differences between the Gly spectra at pH 4.0 (complete deprotonation) between the aqueous (Fig A.1B) and freeze-dried (Fig. A.1D) samples. In the latter case, the $C{=}O_{str}$ (COO^-) vibrational absorption split into two bands which shifted to higher frequencies of 1613±1 and 1637±2 cm^{-1}. The more complex spectrum in the solid state may be due to increased intermolecular interactions between carboxyl and amino groups.

Because such significant effects were observed, it was important to confirm our spectral assignments. In order to do so, we also measured the IR spectra of glycine lyophilized from D_2O at pD 1.1 (Fig A.1E) and pD 4.6 (Fig A.1F) and found that, as predicted, replacement of protons with deuterons only slightly affected those vibrational bands of $C{=}O_{str}$ but dramatically shifted the other bands (now arising from the deuterated amino group) completely out of the spectral region under scrutiny. These findings are consistent with our deduced spectral assignments.

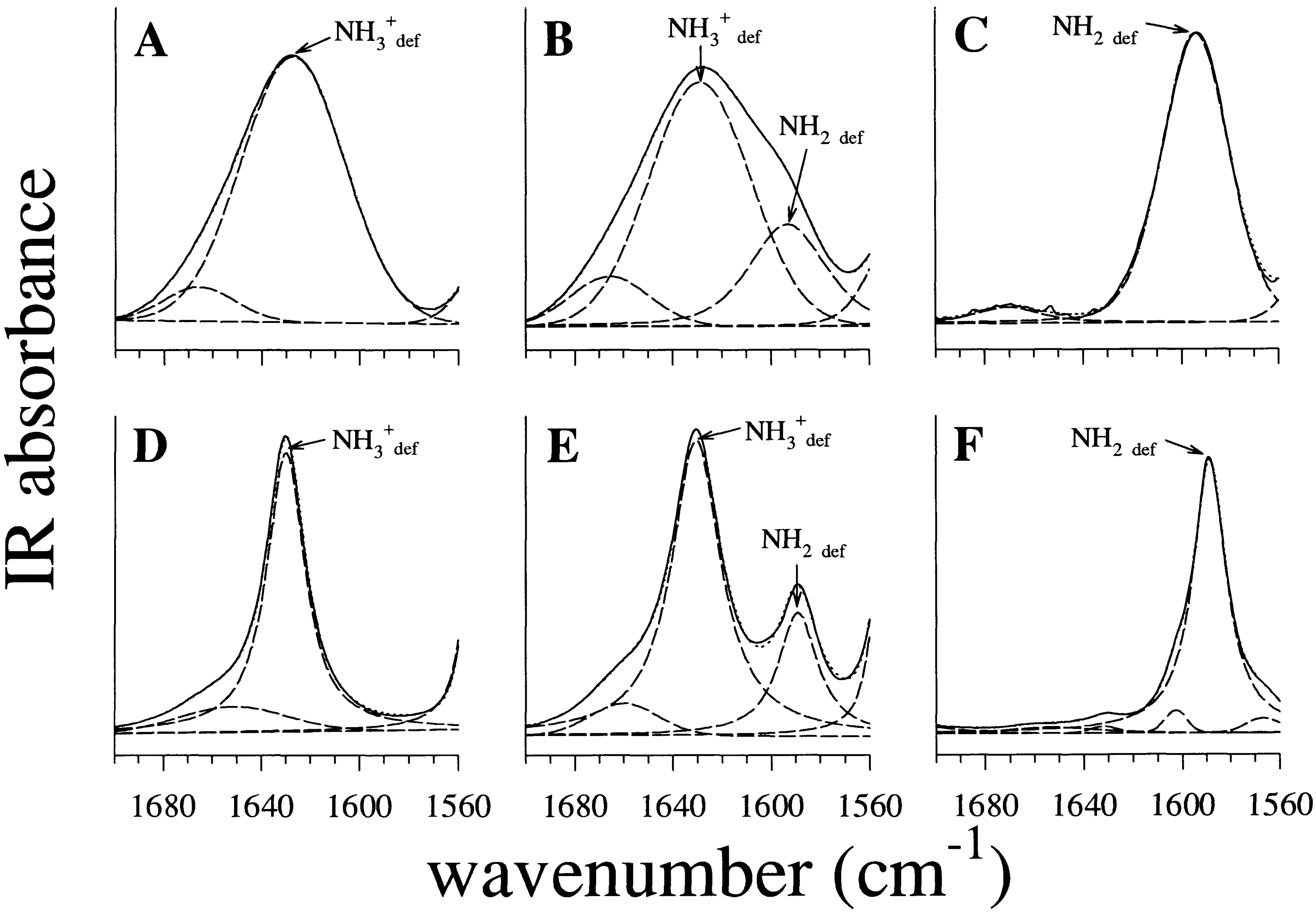
Having assigned wavenumbers for bands corresponding to $C=O_{str}$ for both neutral and ionized species, it was possible to calculate F for lyophilized Gly as a function of pH. The result is shown in Figure A.2B. From these data, it was determined that the "pK_a" in the solid state was 2.57±0.04. Although very similar to the pK_a determined experimentally for the aqueous solution (Fig A.2A), this value is somewhat higher (by some 0.28 units) considering the accuracy of the analysis.

In addition to the carboxyl group, a second ionizable species commonly found in proteins is the amino group. The amino acid Gly contains both such groups, but we were unable to quantitatively investigate the ionization of the amino group (as we did the carboxyl one above) since the spectra in the lyophilized powder became extremely complex upon lyophilization from alkaline pH (data not shown). Therefore, we decided to study a different model compound, Tris, which contains only an amino group.

A portion of the IR spectrum for Tris in water at pH 6.0 (corresponding to essentially complete protonation of the amino group) is depicted in Figure A.3A. The spectral region examined was that in which in the $NH_3^+{}_{def}$ and $NH_2\,{}_{def}$ IR absorption bands are known to occur (Venyaminov and Kalnin, 1990). Of the bands present in this range, the one at 1625±3 was assigned to the protonated amino group, as determined earlier ($NH_3^+{}_{def,as}$ for Gly). Although the minor band occurring at 1670-1660 cm^{-1} is closely overlapping, its continued presence in the spectra under conditions (aqueous solution at pH 10) corresponding to deprotonation (Fig.A.3C) suggests that it does not arise from the amino group. In the latter spectrum, the IR absorption belonging to NH_3^+ disappeared and was replaced by a new peak at 1593±1 cm^{-1}, which was logically assigned to $NH_2\,{}_{def}$. At an intermediate pH of 8.1, peaks corresponding to both $NH_3^+{}_{def}$ and $NH_2\,{}_{def}$ can be distinctly seen (Fig. A.3B).

Unlike the vibrational modes examined for the first case ($C=O_{str}$ in Gly) the magnitude of the vibrations arising from nitrogen-hydrogen bonds are directly impacted by protonation of the amino group of Tris. Hence, it cannot be assumed that the extinction coefficients for the NH_3^+ and NH_2 vibrations are equal. Therefore, we determined their ratio by examination of the IR spectra of Tris in water at pH 8.08, the known pK_a value (Dawson *et al.*, 1986), as proposed above (Section A.2).

Figure A.3 IR absorption of Tris for various liquid and solid samples. In water at (A) pH 6.0, (B) pH 8.1, and (C) pH 10. Powder, lyophilized from water at (D) pH 6.0, (E) pH 8.3, and (F) pH 10.0. Sample concentrations were 200-300 mg/ml for liquids and 1-2 mg per 200 mg KBr for solids.



IR absorbance
A
$NH_3^+{}_{def}$
B
$NH_3^+{}_{def}$
$NH_{2\ def}$
C
$NH_{2\ def}$
D
$NH_3^+{}_{def}$
E
$NH_3^+{}_{def}$
$NH_{2\ def}$
F
$NH_{2\ def}$
1680
1640
1600
1560
wavenumber (cm^{-1})

Employing this strategy, we determined that

$$\int \frac{\varepsilon_{NH_2}}{\varepsilon_{NH_3^+}} = 0.33 \pm 0.04$$

where the term in the numerator refers to IR absorption of $-NH_2$ with the maxima at 1593 cm^{-1} and the denominator refers to IR absorption of $-NH_3^+$ with the maxima at 1625 cm^{-1}. Using this ratio, it was possible to calculate the relative concentrations of protonated and deprotonated Tris molecules using the aqueous spectra obtained over the pH range of 6 to 10. From the data (Fig A.4A), the pK_a for Tris in aqueous solution was experimentally determined as 8.10±0.02, in excellent agreement with the literature value of 8.08 at room temperature (Dawson *et al.*, 1986). This result validates our approach once again, and affirms our value for the extinction coefficient ratio for the protonated and deprotonated species.

Since our goal was to compare this with the pK_a for dehydrated Tris, we next examined the solid spectra for samples lyophilized at various pHs from 6 to 10. We found the absorption bands arising from the $-NH_3^+$ group (1630±1 cm^{-1} in the solid spectrum, pH 6 as depicted in Fig. A.3D) and the $-NH_2$ group (1589±0 cm^{-1} in the solid spectrum at pH 8.3 and 10 as depicted in Figs. A.3E and F, respectively) had the same location as that for the liquid, clearly confirming their assignments. Indeed, the only difference of note is that the IR absorption peaks were more distinct in the freeze-dried powder, a trend also observed for Gly. Note that it was necessary to include some very minor peaks in order to satisfy the criteria of the curve-fitting; these were unassigned and did not factor in our analyses.

Because the IR absorption bands we chose to monitor for the $-NH_3$ and $-NH_2$ species of Tris retained their peak locations (and did not become more complex) upon lyophilization, it was fairly straightforward to calculate F for the samples lyophilized from the various pHs. This plot is depicted in Figure A.4B. From the data, it was determined that the pK_a for Tris in the lyophilized form was 8.34±0.02. This value is 0.24 units higher than the one experimentally determined for Tris in water (Fig. A.4A).

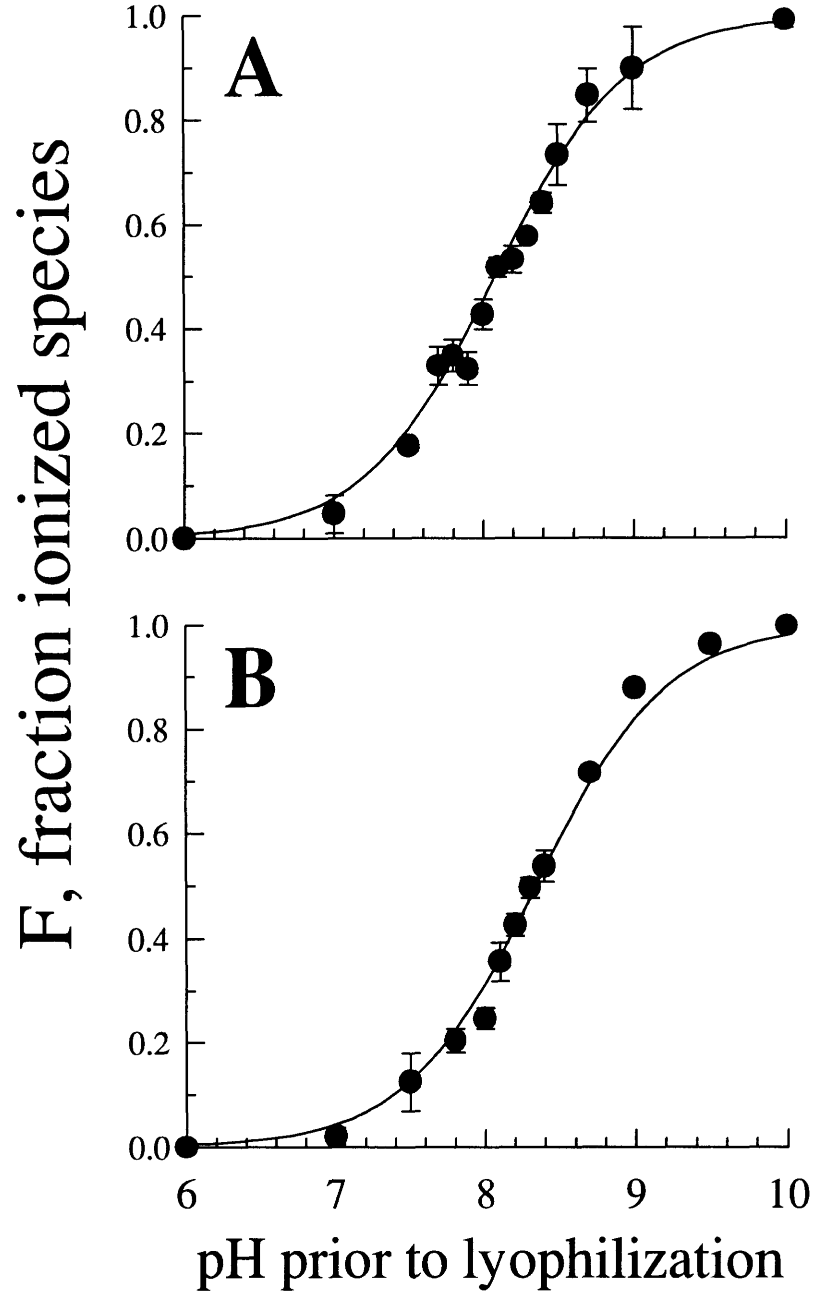


Figure A.4 Determination of midpoint for the ionization of the Tris amino group in the liquid and solid states. (A) The fraction of ionized species in aqueous solution as a function of the pH. The solid line represents the results of the curve fit: $pK_a=8.10\pm0.02$. (B) The fraction of ionized species in the solid state as a function of the pH prior to lyophilization. The solid line represents the results of the curve fit: $pK_a=8.34\pm0.02$. For both liquid and solid determinations, methodologies are discussed in the text.

In addition to carboxyl and amino groups, yet another ionizable species which may be found in proteins is the enol. Thus, we also examined a simple model compound containing this moiety, namely HBA. The IR spectrum for HBA in water at pH 8.5 is shown in Figure A.5A. The region depicted is known to contain enol C–O_{str} vibrations: the peaks located at 1273±2 and 1239±1 cm-1 arise from C–O_{str} (CO^-) and C–O_{str} (COH) vibrations, respectively (Venyaminov and Kalnin, 1990). The additional band located at 1256±2 cm-1 was not assigned. Examination of aqueous HBA spectra obtained at pH 9.8 (Fig. A.5B) and pH 11 (Fig. A.5C) demonstrate that the relative intensities of these peaks shifted as expected from their assignments.

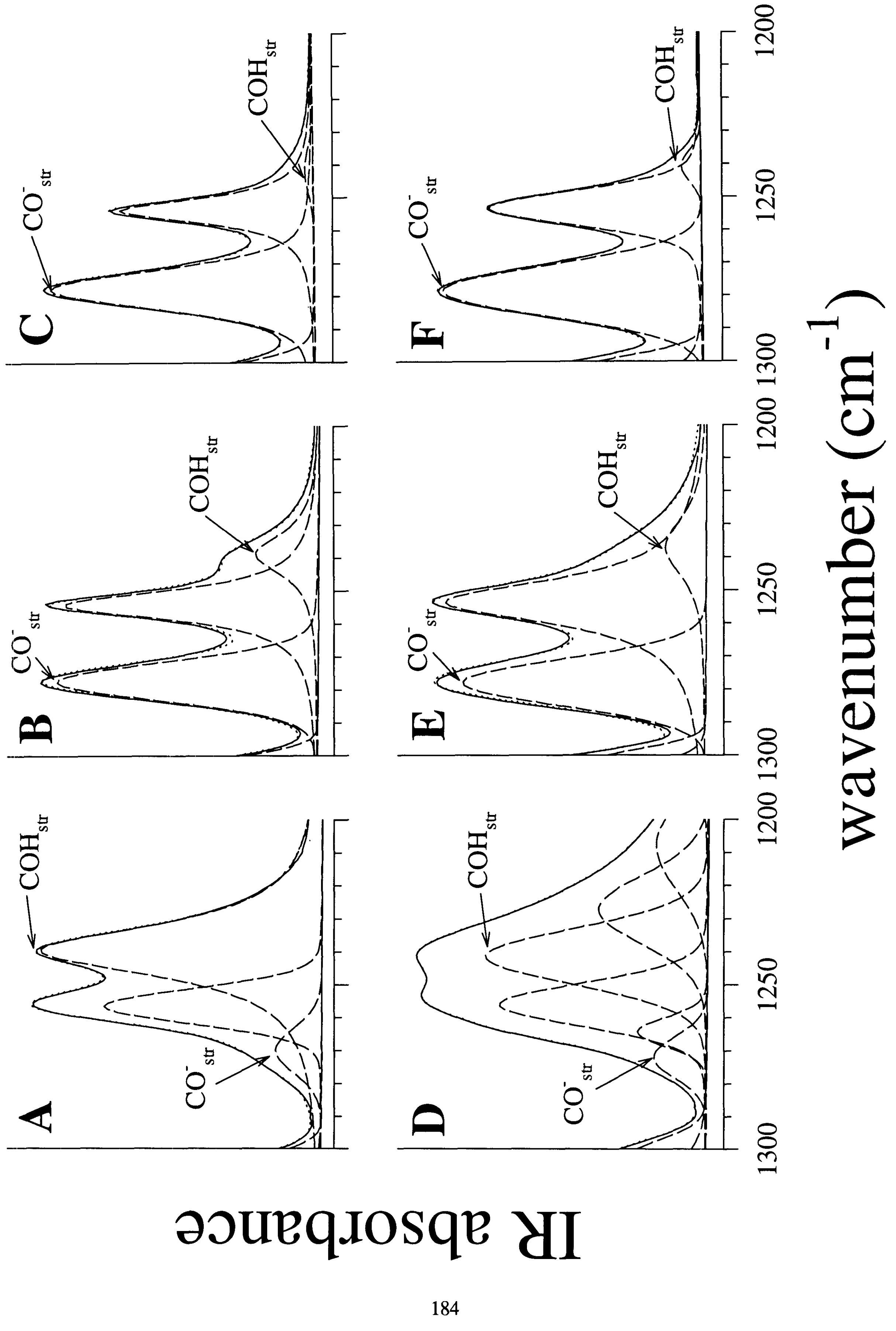
In order to determine the fraction of ionized species, it was necessary to determine the ratio of extinction coefficients the CO^-_{str} and COH_{str} vibrations. To accomplish this, we analyzed the IR spectra of HBA in water at pH 9.85, the known pK_a value (Dean, 1985), employing the approach detailed above (Section A.2). It was found that

$$\int \frac{\varepsilon_{CO^-}}{\varepsilon_{COH}} = 3.1 \pm 0.1$$

where the term in the numerator refers to IR absorption of C–O_{str} (CO^-) with the maxima at 1273 cm^{-1} and the denominator refers to IR absorption due to C–O_{str} (COH) with the maxima at 1239 cm^{-1}. Using this ratio, we were able to calculate the relative concentrations of protonated and deprotonated HBA molecules using the aqueous spectra obtained over the pH range of 8.5 to 11. From the data (Fig A.6A), the pK_a for HBA in aqueous solution was experimentally confirmed as 9.85±0.02.

The final question was: how does the pK_a value for HBA in solution compare to that in the dried state? To answer this question we examined the IR spectra of HBA which was lyophilized from a pH range of 8.5 to 11 (Figs. A.5D-F). At a given pH, the spectra of the solid looked similar to that of the aqueous sample (compare the with Figs. A.5A-C). Thus, it was relatively straightforward to calculate F for the samples of lyophilized HBA (Fig A.6B). From these data, it was determined that the pK_a for HBA in the lyophilized form was 9.88±0.03, identical with the value determined for HBA in aqueous solution (Fig. A.6A).

Figure A.5 IR absorption of HBA for various liquid and solid samples. In water at (A) pH 8.5, (B) pH 9.8, and (C) pH 11.0. Powder, lyophilized from water at (D) pH 8.5, (E) pH 9.8, and (F) pH 11.0. Sample concentrations were 60 mg/ml for liquids and 1-2 mg per 200 mg KBr for solids.


A
B
C
D
E
F
CO^-_{str}
COH_{str}
1300
1250
1200
IR absorbance
wavenumber (cm^{-1})

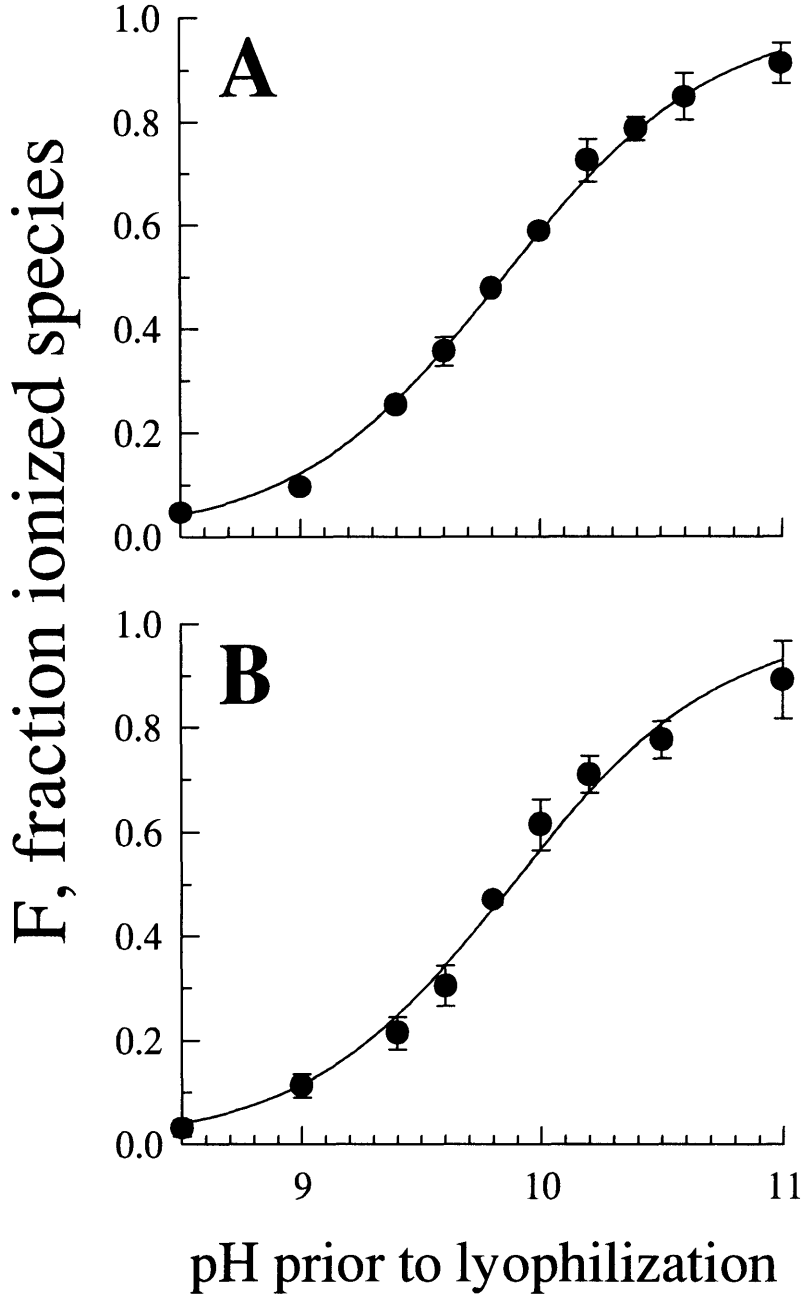


Figure A.6 Determination of midpoint for the ionization of the enol group of HBA. (A) The fraction of ionized species in aqueous solution as a function of the pH. The solid line represents the results of the curve fit: $pK_a=9.85\pm0.02$. (B) The fraction of ionized species in the solid state as a function of the pH prior to lyophilization. The solid line represents the results of the curve fit: $pK_a=9.88\pm0.03$. For both liquid and solid determinations, methodologies are discussed in the text.

A.4 Concluding Remarks

In our study of three model, organic ionizable moieties, the carboxyl group of Gly, the amino group of Tris, and the enol group of HBA, we demonstrated that IR spectroscopy can be employed to determine the midpoint of transition for deprotonation (the pK_a) in both liquid and lyophilized states. For the first two compounds, it was found that this value was very similar, yet slightly (~0.3 units) higher in the solid compared to the aqueous state. In the final case (HBA) it was found that solid and liquid samples had identical pK_a values.

These finding are consistent with previous observations of protein "pH memory" (Klibanov, 1989) and support our hypotheses regarding the dependence of the stability of insulin and rHA on the pH prior to lyophilization. However, one must exercise some caution in extrapolating the results obtained with model compounds to proteins since proteins have three-dimensional structures (which profoundly influence the pK_a of individual residues) which may be perturbed upon lyophilization (Griebenow and Klibanov, 1995). Therefore, it is conceivable that the pK_a of an individual residue may be altered by this rearrangement in protein structure, rather than the removal of water itself.