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Research Article

Moisture-Induced Aggregation of Lyophilized Insulin

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A critical problem in the storage and delivery of pharmaceutical proteins is aggregation in the solid state induced by elevated temperature and moisture. These conditions are particularly relevant for studies of protein stability during accelerated storage or for proteins loaded in polymeric delivery devices *in vivo*. In the present investigation, we have found that, when exposed to an environment simulating these conditions, lyophilized insulin undergoes both covalent and noncovalent aggregation. The covalent process has been elucidated to be intermolecular thiol-catalyzed disulfide interchange following β-elimination of an intact disulfide bridge in the insulin molecule. This process is accelerated by increasing the temperature and water content of the insulin powder or by performing lyophilization and/or dissolution of insulin in alkaline media. The aggregation can be ameliorated by the presence of Cu^{2+}, which presumably catalyzes the oxidization of free thiols. The water sorption isotherm for insulin reveals that the extent of aggregation directly correlates with the water uptake by the lyophilized insulin powder, thus pointing to the critical role of protein conformational mobility in the aggregation process.

KEY WORDS: insulin; lyophilization; aggregation; β-elimination; disulfide interchange.

INTRODUCTION

Modern biotechnology has made feasible the production of recombinant proteins for a variety of therapeutic and diagnostic applications. For example, there are over 100 genetically engineered proteins currently in Phase I clinical trials (1). The successful exploitation of these pharmaceutical proteins requires preservation of biological activity in all steps of development from purification to storage to administration. Compared to traditional drugs, proteins pose many additional challenges in preserving activity because they have higher molecular weights, contain multiple functional groups, and possess exquisite three-dimensional structures (2). The relatively fragile nature of most proteins is an obstacle to their processing, storage, and delivery (3,4). An example is the hormone insulin which exhibits instability in both solution and suspension (5).

The stability of solid pharmaceutical proteins must also be considered in the development of controlled delivery systems, such as sustained release from polymeric matrices. (This delivery system has been proposed for pharmaceutical proteins which demonstrate short biological half-lives when administered orally and thus require frequent bolus injections.) 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 as it is exposed to elevated levels of temperature and moisture (6).

During the transition from a dry solid protein (with bulk water removed) to the fully hydrated one, profound changes are observed in protein conformation, dynamics, and other properties that have repercussions for stability in the solid state (7). 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 (8). The hydration of solid proteins leads to increased rates of deleterious processes due to greater conformational flexibility of the protein molecule and higher mobility of reactive species (3).

Although the role of moisture in solid-state stability of low molecular weight drugs is well established (9), there have been few studies of the effect of moisture on the stability of solid pharmaceutical proteins. Such an investigation of the model protein bovine serum albumin revealed its moisture-induced aggregation due to thiol–disulfide interchange (10). In addition, moisture-induced deterioration of solid pharmaceutical proteins has been reported, such as aggregation of lyophilized tissue-type plasminogen activator (tPA) (11) and growth hormone (3,12). Mechanistic understanding of such processes, which is the gist of the present study, should lead to the development of rational stabilization strategies and hence to more stable pharmaceutical protein formulations.

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EXPERIMENTAL

Materials

Bovine Zn-insulin was purchased from Sigma Chemical Co. Human Zn-free insulin was a generous gift from Eli Lilly & Co. All other biochemicals, inorganic salts, and other chemicals were of analytical grade or purer and were obtained from commercial suppliers.

Preparation of Insulin Samples

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 the addition of approximately 13 mL of 0.04 *M* KOH containing 10 m*M* EDTA. The resulting insulin precipitate was collected by centrifugation at 5000 × *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 with a 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 hr 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 stated otherwise. Insulin samples were frozen in liquid nitrogen and lyophilized on a Labconco Freeze Drier 8 at a pressure of approximately 10 μm Hg and −50°C for at least 48 hr. Insulin dissolved at 0.05 mg/mL by stirring at 37°C and pH 10 for 2 hr, and finally adjusted to pH 7.3 prior to lyophilization, is referred to here as "rapidly aggregating" insulin. The average water content of all lyophilized insulin samples was 6.3 ± 1.8% (w/w) as determined by Karl Fischer titration (see below).

Moisture-Induced Aggregation

To study the effect of moisture upon solid insulin stability, we devised the following experimental approach. Lyophilized protein powder (1.0 mg) was weighted in a 10-mL beaker. The beaker was then placed into a desiccator at a constant relative humidity, which was achieved by the presence of an aqueous saturated salt solution in a container within the desiccator (see below). The sealed desiccator was kept at a constant temperature inside a VWR 1330 FD oven at 50°C, unless stated otherwise. A septum port on the desiccator lid allows on-line measurements of temperature and relative humidity inside the desiccator with a VWR digital hygrometer/thermometer. Following the desired length of incubation, the wetted protein powder was removed, and 5 mL of phosphate-buffered saline (PBS; 5 m*M* sodium phosphate, pH 7.3, and 150 m*M* NaCl) was added to the beaker and stirred for 2 hr at 37°C to ensure dissolution of the soluble fraction of the sample. All samples were then filtered through a Millex-GV filter (Millipore) of 0.22-μm pore size to remove insoluble materials. Standards used for protein determinations were also treated in this manner, except they were not incubated in the chamber.

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. For all these assays, unincubated insulin from the same batch was used as a protein standard. All spectrophotometric measurements were conducted in a Hitachi U-3110 spectrophotometer.

Determination of Free Thiols

Free thiols were measured using Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which reacts rapidly and quantitatively with the thiolate ion to yield the 2-nitro-5-thiobenzoate anion (TNB^{2-}), which has an absorption maximum at 412 nm (13). Insulin was dissolved at 2 mg/mL by stirring for 2 hr at 37°C in 100 m*M* sodium phosphate (pH 7.3) and 1 m*M* EDTA and then filtered through a 0.22-μm Millex-GV filter. Varying amounts of the insulin solution were added to the reaction mixture, which had a final DTNB concentration of 0.3 m*M*, and the free thiol concentration was calculated from the absorbance of the reaction mixture at 412 nm (13). To determine whether the free thiols had a low molecular weight (e.g., hydrosulfide ion) or were protein-bound, insulin was concentrated approximately threefold by loading the sample on an Amicon Centricon-3 microconcentrator (with a 3000-Da molecular weight cutoff) and centrifuging the microconcentrator for 1 hr at 5000 × *g*. 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.

Determination of 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.) with a Mettler DL18 autotitrator. This method has been routinely used to measure the water content of protein powders (14).

Samples (2 mg each) were stored at various relative humidities until equilibrium uptake of water by the lyophilized powder was achieved and no additional water was absorbed (7 days at 25°C or 6 hr at 50°C). Such rapid absorption of water has been observed for other proteins (15). Various relative humidities were achieved by the use of the following saturated salt solutions in water (16,17): LiCl, 11% at 25° and 50°C; $MgCl_2$, 31% at 50°C; NaBr, 51% at 50°C; $NaNO_2$, 64% at 25°C; KI, 65% at 50°C; $NaNO_3$, 69% at 50°C; NaCl, 75% at 25°C and 74% at 50°C; KCl, 84% at 25°C and 81% at 50°C; KNO_3, 85% at 50°C; $BaCl_2$, 88% at 50°C; and K_2SO_4, 97% at 25°C and 96% at 50°C.

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 sec (Ultrasonic Cleaner Model 250, RAI Research) and injected into the Fis-

cher titrator. All water contents are reported as percentages (w/w).

Modeling of the Water Sorption Isotherm

The water vapor sorption isotherm for rapidly aggregating insulin was modeled using the Brunauer–Emmett–Teller (BET) gas adsorption equation (18):

$$M = \frac{aM_0C}{(1 - a)(1 - a + Ca)}$$

where M is the equilibrium moisture content of the protein (g of water/100 g of dry protein), a is the activity of the sorbed water (which is equal to the % relative humidity), C is a constant related to the heat of absorption of the monolayer, and M_0 represents the monolayer coverage of water (g of water/100 g of 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 $a/[(1 - a)M]$ (BET plot). The BET model has been used extensively to describe the water vapor sorption isotherm and monolayer water sorption for proteins (11,15,19).

RESULTS AND DISCUSSION

The stability of the insulin molecule in various formulations is an important issue in insulin-dependent diabetes therapy. In particular, aggregation of insulin is known to lead to severely reduced biological activity and increased immunogenicity (5). In addition, the aggregation of insulin suspended in a polymeric matrix within a controlled-release formulation may lead to lowered release rates. Thus, we have focused this study on the moisture-induced aggregation of lyophilized insulin. Our experimental approach 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 formulation in the wetted protein.

Aggregation of Insulin Dissolved at Acidic pH

A variety of insulin samples was prepared to test for 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 (see Experimental for details).

To simulate accelerated storage conditions, we initially investigated the stability of lyophilized bovine insulin at a 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 shown in Fig. 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.

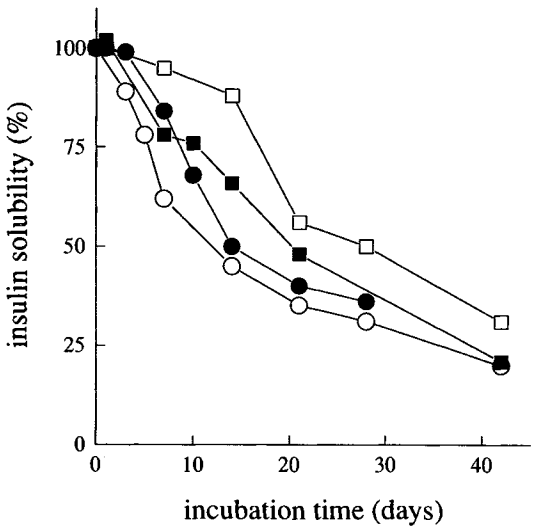


Fig. 1. Aggregation of lyophilized insulin at 50°C and 96% relative humidity. Zn-insulin was dissolved by stirring in aqueous solution at pH 3.0 for 20 min, and then the pH was adjusted to 7.3. Insulin concentrations prior to lyophilization were (○) 0.05 mg/mL Zn-insulin, (●) 0.15 mg/mL Zn-insulin, (□) 1.0 mg/mL Zn-insulin, and (■) 1.0 mg/mL K-insulin. For conditions, see Experimental.

To make stability comparisons between samples, formation of insoluble aggregates was measured at fixed conditions and lengths of incubation time. To this end, the insulin samples in Fig. 1 were incubated at 50°C and 96% relative humidity for 1- and 2-week periods. The resulting losses in protein solubility are shown in Fig. 2. It is seen that there is

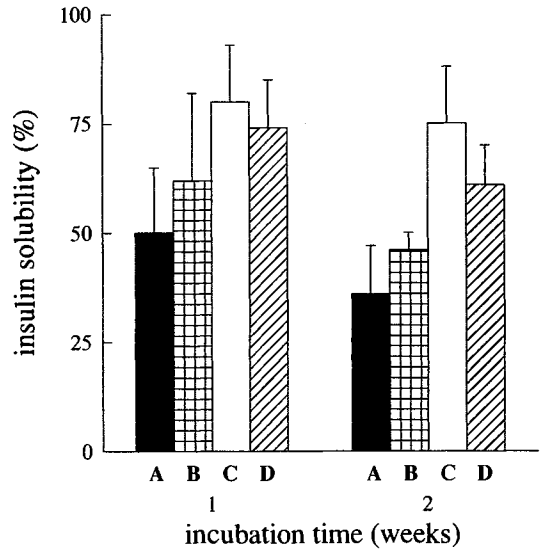


Fig. 2. Insulin aggregation following 1- and 2-week incubations at 50°C and 96% relative humidity. Zn-insulin was dissolved by stirring in aqueous solution at pH 3.0 for 20 min, and then the pH was adjusted to 7.3. Insulin concentrations prior to lyophilization were (A) 0.05 mg/mL Zn-insulin, (B) 0.15 mg/mL Zn-insulin, (C) 1.0 mg/mL Zn-insulin, and (D) 1.0 mg/ml K-insulin. Error bars represent the standard deviation of three data points. For conditions, see Experimental.

no clear dependence of the degree of aggregation on insulin concentration prior to lyophilization. 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 (20), which, in solution, results in less stable insulin preparations (5). Under these conditions insulin molecules may also adsorb at the hydrophobic air–water interface resulting in denaturation and aggregation (21).

Next, to investigate the nature of the moisture-induced insulin aggregates obtained, we attempted to redissolve the aggregated protein in a number of solutions. The results obtained are given in Table I. 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 noncovalent forces—the denaturants would disrupt only the latter. When placed in a solution of a thiol reagent, 10 m*M* dithioerythritol, and 1 m*M* EDTA to prevent its autooxidation, 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 noncovalent interactions and also by intermolecular disulfide linkages.

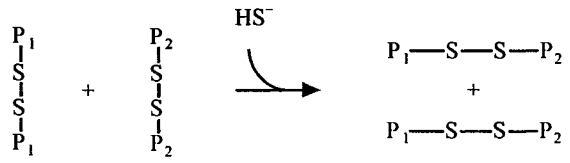
The formation of covalent and noncovalent insulin aggregates has been observed in other insulin systems. In agitated solutions with hydrophobic interfaces, insulin exhibits noncovalent aggregation initiated by denaturation (22). Long-term stability studies of various insulin therapeutic preparations (solutions and suspensions) also revealed covalent dimers and soluble oligomers formed over time via intermolecular disulfide bonds (23–25). In addition, aggregation of solid, crystalline Zn-insulin has been observed at elevated temperatures due to an undetermined mechanism (26).

Table I. Solubility of Moisture-Induced Aggregates of Lyophilized Insulin in Various Solutions[a]

Solvent	Insulin aggregate solubility (%)
PBS	0
PBS + 1 m*M* EDTA	0
PBS + 10 m*M* dithioerythritol (DTE) + 1 m*M* EDTA	21
PBS + 6 *M* guanidine hydrochloride	56
PBS + 8 *M* urea	50
PBS + 8 *M* urea + 10 m*M* DTE + 1 m*M* EDTA	104
PBS + 6 *M* guanidine hydrochloride + 10 m*M* DTE + 1 m*M* 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 m*M* sodium phosphate, pH 7.3, and 150 m*M* 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 hr) 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. For further details, see Experimental. Note that separate calibration curves were obtained for protein determinations in the presence of the additives listed.

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 (25,27). 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. These free thiols can subsequently catalyze the reshuffling of intact protein disulfides, thereby leading to intermolecular disulfide cross-links:



where P_1 and P_2 represent two separate protein molecules. In a study of disulfide exchange in neutral insulin solutions and suspensions, Brange (25) has suggested that the A7–B7 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 (25).

Because of the involvement of hydroxide ions, β-elimination accelerates under alkaline conditions. Lyophilized protein powders exhibit "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 (28). 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 (24). In the present study, a similar trend was observed experimentally for lyophilized insulin, as shown in Fig. 3. For example, insulin lyophilized at a concentration of 1 mg/mL from pH 10 exhibits complete aggregation in just 1 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 3 weeks.

Aggregation of Insulin Dissolved at Alkaline pH (Rapidly Aggregating Insulin)

If the covalent aggregation is initiated by β-elimination,

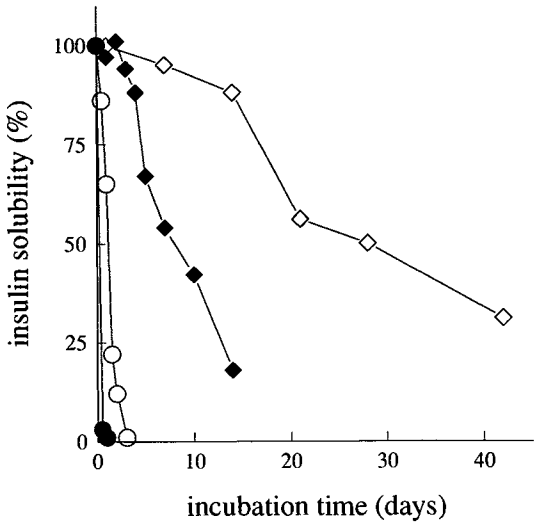


Fig. 3. Dependence of the moisture-induced aggregation of lyophilized insulin on the pH of the aqueous solution prior to lyophilization. Insulin was dissolved at 1.0 mg/mL by stirring at pH 3.0 for 20 min, and the pH was subsequently adjusted to varying values: (◇) 7.3, (◆) 8.0, (○) 9.0, and (●) 10.0. These samples were incubated at 50°C and 96% relative humidity. For conditions, see Experimental.

then insulin dissolved at alkaline pH should exhibit increased aggregation due to the formation of free thiols in aqueous solution *prior* to lyophilization (27). 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-hr 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 under the same conditions and subjected to the same environment: after 1 month of incubation the loss of solubility was less than 75% (open circles in Fig. 1). 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 that transpired during the alkaline dissolution process, presumably the β-elimination reaction. Using Ellman's reagent, DTNB (see Experimental for details), 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 the source of insulin, as well as with the biological activity and composition of the insulin formulation (23). Thus, it is important to select downstream processing conditions judiciously prior to storage and delivery of insulin and other pharmaceutical proteins.

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 as for acid-dissolved insulin in Table I. The results from the dissolution analysis of the moisture-induced aggregates of rapidly aggregating insulin are given in Table II. 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 noncovalent route that the latter mechanism is no longer appreciable.

To determine whether free thiols formed as a result of a process occurring *in the solid state,* rapidly aggregating insulin was incubated for 24 hr 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 of thiol/initial mol of insulin. (This is the average value and standard deviation of four separate determinations using two 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 concentrations 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.

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 and 45°C, and the data were compared to those obtained at 50°C. The rate of insulin aggregation was found to drop precipitously with temperature: aggregation is

Table II. Solubility of Moisture-Induced Aggregates of the Rapidly Aggregating Insulin Sample in Various Solutions[a]

Solvent	Insulin aggregate solubility (%)
PBS	0 ± 8
PBS + 8 *M* urea	3 ± 10
PBS + 10 m*M* dithioerythritol (DTE) + 1 m*M* EDTA	122 ± 7
PBS + 8 *M* urea + 10 m*M* DTE + 1 m*M* 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 hr at 37°C and pH 10. Samples were incubated for 24 hr at 50°C and 96% relative humidity. For other procedures, see Table I, footnote *a*.

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 (5).

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) preformed 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 (Fig. 4). All samples were prepared in the same manner as rapidly aggregating insulin, except the final pH was varied between 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.

In addition to lowering the formation of thiols, another approach to stabilize insulin is to transform the free thiols chemically to a species nonreactive toward disulfides. Free thiols can be oxidized by molecular oxygen in the presence of certain transition metal ions (29). 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 at a mole ratio (cation to insulin) of 1:10. Because they were added after dissolution, any effect imparted is a result of processes occurring during incubation of the solid, and not during the dissolution prior to lyophilization. These samples were incubated for 24 hr at 50°C and 96% relative humidity, and the resultant solubility losses were compared with that of insulin prepared without added metal ions (Fig. 5). As a negative control, $CaCl_2$ was added to one formulation, which 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.

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 stabilize the rapidly aggregating insulin completely 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 terms of rates of catalysis and that cupric ion is the foremost of these (29). 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.

Yet another approach to stabilization of insulin in the solid state is controlling the humidity. This line of investiga-

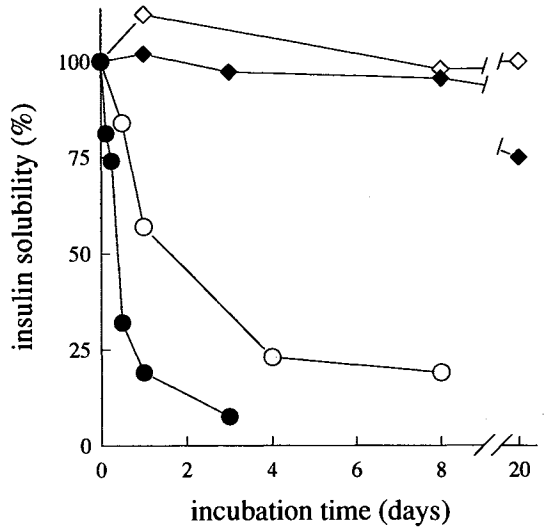


Fig. 4. Dependence of the moisture-induced aggregation of lyophilized insulin on the pH of the aqueous solution prior to lyophilization. Rapidly aggregating insulin was prepared and the pH was subsequently adjusted to (◇) 4.5, (◆) 6.5, (○) 7.3, or (●) 8.5. Samples were incubated at 45°C and 75% relative humidity. For conditions, see Experimental.

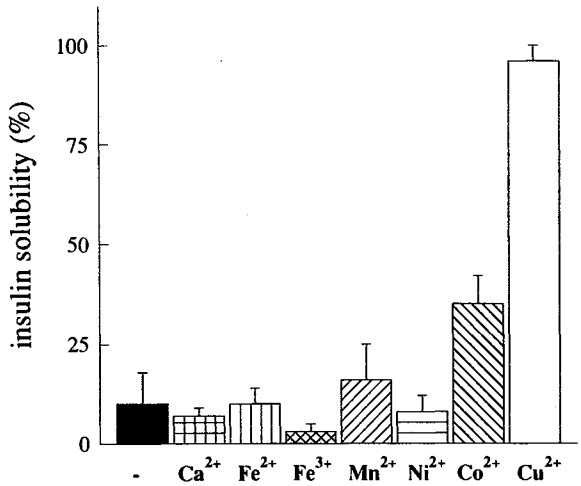


Fig. 5. Effect of addition of divalent metal ions on solid-state stability of insulin. Rapidly aggregating insulin was prepared in the absence (−) and presence of various metal chlorides, as listed. The metal ion-to-insulin mole ratio was 1:10. Depicted is the insulin solubility remaining following a 24-hr incubation at 50°C and 96% relative humidity. The error bars represent the standard deviation of three data points. For conditions, see Experimental.

tion 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 protein degradation reactions (3,10–12). It follows that if water is involved in these processes as a reactant, as a mobile phase for reactants, or as a molecular lubricant (3), 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. Rapidly aggregating insulin was incubated at 50°C and a number of relative humidities, ranging from 11 to 96%. As shown in Fig. 6, the fastest formation of insoluble aggregates was observed at relative humidities of 75% and above.

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 Fig. 7A 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 (data not shown). The average water content of all samples following incubation at 11 through 65% relative humidity is 5.8 ± 1.7%, i.e., equivalent to the water content of the unincubated sample (5.0 ± 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 (Fig. 7B). Thus, as predicted, the aggregation rate increased markedly with increasing sorbed water.

The water sorption isotherm for insulin was modeled using the BET equation, as depicted by the solid curve in Fig. 7A. The BET isotherm has been used extensively for proteins (11,15,19), and our experimental data for insulin exhibited a fair fit to this model (the correlation coefficient was 0.84 for the BET plot; see Experimental for details). The BET model was also useful in calculating a theoretical monolayer water coverage of the insulin molecule. From the data presented in Fig. 7A, this value was calculated to be approximately 4.4%. In reality, this water does not exist as a contiguous monolayer surrounding the protein surface, but is clustered at the polar sites on the protein surface (7).

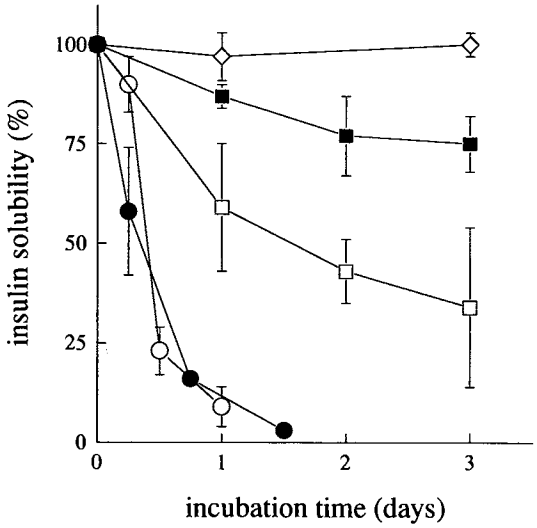


Fig. 6. Moisture-induced aggregation of lyophilized insulin as a function of relative humidity (rh). Rapidly aggregating insulin was prepared and incubated at 50°C inside humidified chambers containing the following saturated aqueous salt solutions: (●) K_2SO_4 (96% rh), (○) NaCl (74% rh), (□) $NaNO_3$ (69% rh), (■) KI (65% rh), and (◇) LiCl (11% rh). Error bars represent the standard deviation of at least three data points. For conditions, see Experimental.

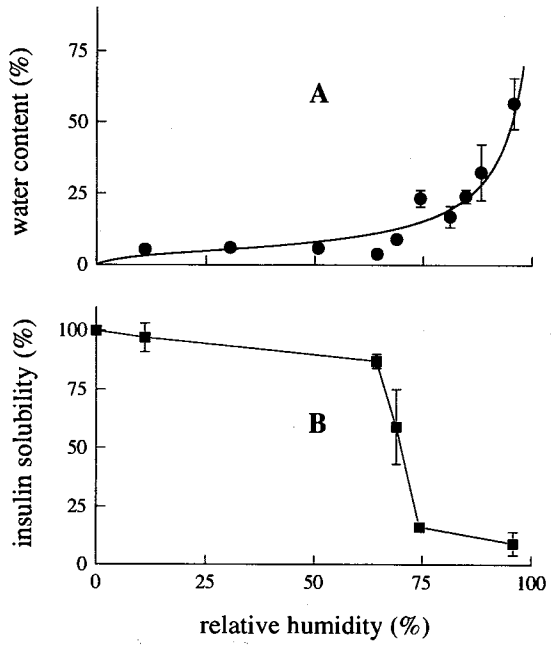


Fig. 7. Lyophilized insulin aggregation as a function of water sorption of the protein. (A) The sorption isotherm for the rapidly aggregating insulin sample determined at 50°C. Error bars represent the standard deviation of at least three data points. The solid curve represents the fit to the BET isotherm (see Experimental) using the parameters M_0 = 4.6 g water/100 g protein (4.4%, w/w) and C = 13.6. (B) The aggregation of rapidly aggregating insulin following incubation for 24 hr at 50°C at various relative humidities. For conditions, see Experimental.

The monolayer water coverage was also estimated using a hypothesis proposed by Pauling (30) and Green (31), which predicts water sorption monolayers in good agreement with experimental data (11). According to this hypothesis, protein side chains can be divided into strong water-binding sites, weak water-binding sites, and sites with negligible water-binding capacity. Any additional water sorbed on the protein forms multilayers. This model makes the assumption 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, 1 Thr, and the 2 carboxy termini of the A and B chains. Accordingly, the hypothesis predicts 14 mol-equiv. of water bound to strong water-binding sites, which corresponds to 4.2% water at a monolayer coverage. This is in agreement with the value of 4.4% obtained from the BET. Taking the Pauling and Green hypothesis a step further, one can examine the weak water-binding residues in the insulin molecule: 3 Asn, 3 Gln, 2 His,

1 Lys, and 1 Arg. Accordingly, if all strong and weak water-binding sites were occupied, the water content would be 7.0%. Since the insulin used to determine the sorption isotherm in Fig. 7A actually had a water content of 5.0 ± 0.9%, we conclude that all the strong water-binding sites and some of the weak ones contained sorbed water. It follows, then, that any additionally sorbed water will form multilayers, a conjecture which would lead to a water sorption isotherm where multilayer water sorption dominates. Such an isotherm would exhibit a trend similar to that observed for insulin.

Concluding Remarks

Finally, we also investigated the behavior of solid recombinant human insulin, a common source of insulin for therapeutic use (5). Samples of Zn-free human insulin were prepared by dissolution under acidic conditions (stirring at pH 3.0 for 20 min) and adjustment of 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 Fig. 8. The data demonstrate a pattern of aggregation similar to those of bovine insulin samples: the aggregation rate increases with pH prior to lyophilization. Based on the structural similarity of bovine and human insulin, which differ by only three amino acid residues (5), it is reasonable to assume that human insulin undergoes the same mechanisms of aggregation as its bovine counterpart.

In summary, the formation of insoluble aggregates in lyophilized insulin at an elevated temperature and a high humidity has been investigated and mechanistically rationalized for the first time. The aggregates are formed via two distinct mechanisms which occur in the solid state: noncovalent interactions and β-elimination of cystines, followed by thiol-catalyzed disulfide interchange. On the basis of our findings, rational approaches for stabilization of insulin have been proposed.

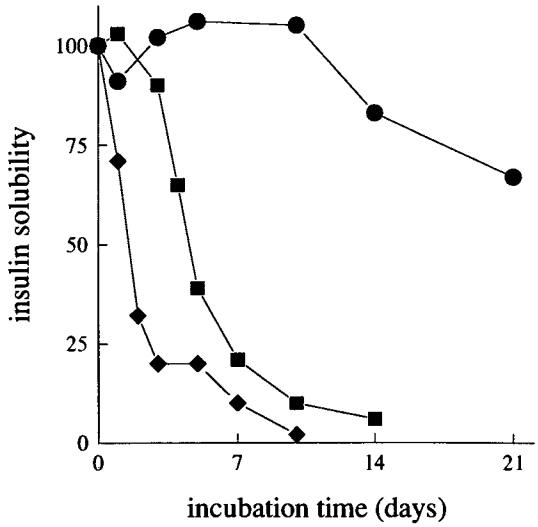


Fig. 8. Moisture-induced aggregation of lyophilized Zn-free human insulin at 50°C and 96% relative humidity. Insulin was dissolved at 1.0 mg/mL by stirring in aqueous solution at pH 3.0 for 20 min, and the pH was subsequently adjusted to varying values: (●) 6.5, (■) 7.3, and (◆) 8.5. For conditions, see Experimental.

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