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Research Article

Solid-State Stability of Human Insulin I. Mechanism and the Effect of Water on the Kinetics of Degradation in Lyophiles from pH 2–5 Solutions

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Purpose. Previous studies have established that in aqueous solution at low pH human insulin decomposition proceeds through a cyclic anhydride intermediate leading to the formation of both deamidated and covalent dimer products. This study examines the mechanism and kinetics of insulin degradation in the amorphous solid state (lyophilized powders) as a function of water content over a similar pH range.

Methods. Solutions of 1.0 mg/mL insulin were adjusted to pH 2–5 using HCl, freeze-dried, then exposed to various relative humidities at 35°C. The water content within the powders was determined by Karl Fischer titration, and the concentrations of insulin and its degradation products were determined by HPLC. Degradation kinetics were determined by both the initial rates of product formation and insulin disappearance.

Results. Semi-logarithmic plots of insulin remaining in lyophilized powders versus time were non-linear, asymptotically approaching non-zero apparent plateau values, mathematically describable by a reversible, first-order kinetic model. The rate of degradation of insulin in the solid state was observed to increase with decreasing apparent pH ('pH') yielding, at any given water content, solid-state 'pH'-rate profiles parallel to the solution pH-rate profile. This 'pH' dependence could be accounted for in terms of the fraction of the insulin A21 carboxyl in its neutral form, with an apparent pKa of ≈4, independent of water content. Aniline trapping studies established that the mechanism of degradation of human insulin in lyophilized powders between pH 3–5 and at 35°C involves rate-limiting intramolecular nucleophilic attack of the Asn_{A21} C-terminal carboxylic acid onto the side-chain amide carbonyl to form a reactive cyclic anhydride intermediate, which further reacts with either water or an N-terminal primary amino group (e.g., Phe_{B1} and Gly_{A1}) of another insulin molecule to generate either deamidated insulin (Asp_{A21}) or an amide-linked covalent dimer (e.g., [Asp_{A21}-Phe_{B1}] or [Asp_{A21}-Gly_{A1}]), respectively. The rate of insulin degradation in lyophilized powders at 35°C increases with water content at levels of hydration well below the suspected glass transition and approaches the rate in solution at or near the water content (20–50%) required to induce a glass transition.

Conclusions. The decomposition of human insulin in lyophilized powders between pH 3–5 is a water induced solid-state reaction accelerated by the plasticization effect of sorbed water. The formation of the cyclic anhydride intermediate at A21 occurs readily even in the glassy state, presumably due to the conformational flexibility of the A21 segment even under conditions in which the insulin molecules as a whole are largely immobile.

KEY WORDS: protein stability; solid-state degradation; deamidation; covalent dimerization; acyl transfer; intramolecular catalysis.

INTRODUCTION

The inherent chemical and/or physical instability of many proteins in aqueous solution often necessitates their formulation as lyophilized powders to attain an acceptable shelf-life (1–4). However, the extent to which the stability of a given protein is improved in the lyophilized solid state depends on a variety of important variables including the residual moisture, temperature, the viscoelastic state of the amorphous solid, the excipients present, and the nature of the degradation pathway (5–9). The dependence of protein decomposition on residual moisture is particularly complex because water simultaneously may act not only directly as a reactant in hydrolysis and deamidation reactions, but also as a medium for reactant mobilization, as a proton transfer agent, and as a plasticizer to increase conformational motions within the system (5).

The "rigidification" of amorphous solids accompanying water removal and/or decreasing temperature is widely held to be important in the stabilization of lyophilized proteins. In the glassy state well below the glass transition temperature, T_g, of

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a material, molecular mobility is extremely limited (10,11). Increases in mobility accompanying the reduction of T_g to the storage temperature with increases in water content are implicated as a crucial factor in a variety of solid-state reactions including the recrystallization of small molecules in amorphous solids (12–16), the onset of enzyme activity (17), and other solid-state chemical reactions (18). Molecular mobility is not negligible, however, even at temperatures well below the glass transition temperature (19), and protein decomposition may occur below T_g albeit at slower rates (6).

Although the sensitivities of specific degradation pathways in the solid state to moisture have not been explored to a sufficient extent to allow generalizations, this has been a topic of recent interest. Thus, Hageman *et al.* suggested that intermolecular reactions may be preferentially accelerated in the solid state due to the high "effective concentration" of proteins (20). Oliyai and Borchardt found that cyclic imide formation was increasingly favored over peptide hydrolysis at low moisture levels in the solid state (21). Roy *et al.*, on the other hand, noted equivalent effects of moisture-induced mobility changes on three different decomposition reactions in a monoclonal antibody-vinca alkaloid conjugate (9).

The decomposition of human insulin in lyophilized powders prepared from acidic solution formulations appears to provide a unique opportunity to examine in greater depth the sensitivity of various reaction pathways to moisture and the changes in molecular mobility and conformational flexibility accompanying increases in hydration. Studies in these laboratories have previously provided a detailed mechanism of the degradation of human insulin in solution between pH 2–5 at 35°C (22–24). Under these conditions, both deamidation and covalent dimerization at the C-terminal Asn_{A21} occur via rate-limiting intramolecular nucleophilic attack of the neutral (protonated) C-terminal carboxylic acid onto the side-chain amide forming a reactive cyclic anhydride intermediate which quickly reacts with either water to form [$desamido_{A21}$] insulin, or with the free amino at the N-terminal Phe_{B1} of another molecule of insulin to form the [Asp_{A21}-Phe_{B1}] covalent amide linked dimer. Intramolecular formation of a cyclic intermediate requires only localized conformational flexibility in order for the reactive centers to come into contact. If this mechanism accounts for the decomposition of insulin in lyophilized solids over the same 'pH' range and if formation of the anhydride intermediate is rate-limiting, one might anticipate that reaction may continue in the glassy state at appreciable rates because only local molecular motions are involved. Partitioning of the cyclic intermediate to either deamidated insulin or covalent aggregates would seemingly require molecular mobility of at least one of the reactants (i.e., water in the case of deamidation and the insulin molecule in the case of covalent aggregate formation). The diffusivity of water and proximity of water molecules to the site of reaction might favor deamidation while the high "effective concentration" of insulin at low water contents might favor covalent aggregate formation if sufficient mobility exists.

This study examines the mechanism of solid-state decomposition of human insulin in lyophilized formulations prepared from solutions having the same pH as those examined previously in establishing the solution mechanism (22–24). The kinetics of degradation were obtained as a function of both 'pH' and water content and in the presence and absence of aniline (used as an anhydride trapping reagent) to further characterize the nature of the reaction, identify the rate-limiting step and test the hypothesis that sufficient conformational flexibility may exist for this intramolecular nucleophilic reaction to proceed even at relatively low water contents.

MATERIALS AND METHODS

Materials

Recombinant human insulin, obtained as the zinc containing solid Pentex® (Miles Inc., Kankakee, IL) was converted to the zinc-free neutral lyophilized solid as previously described (22). The resulting amorphous white powdery solid was stored desiccated at 5°C until further use. Purity by RP-HPLC was >99% with the major impurity being [$desamido_{A21}$] insulin with trace amounts of covalent dimers. Karl Fischer moisture determinations of this solid yielded an average water content of 5.4%. All reagents were analytical grade and used as supplied. Chromatographic solvents were HPLC grade.

Lyophile Preparation and Storage

A 1.0 mg/mL suspension of zinc-free human insulin in deionized water was adjusted to pH 5.0 using a pH meter (PHM82, Radiometer America, Cleveland, OH) equipped with a Ross combination electrode (Orion Research Inc. Boston, MA) using 1.0 M HCl with constant stirring. Aliquots (1.0 mL) of this suspension were transferred to 4 ml glass vials. The pH of the remaining suspension was then further adjusted to pH 4.0, 3.0 or 2.0 with 1.0 M HCl to obtain clear solutions and, at each pH value, aliquots of the solution were transferred to 4 ml glass vials. In another procedure, the pH was first adjusted to 2.0 with 1.0 M HCl, then back-adjusted with 1.0 M NaOH to pH 3.0, 4.0 and 5.0. Using the latter procedure, the powder contained ≈0.01 mmol NaCl per mg of human insulin after lyophilization.

A typical lyophilization cycle was initiated by placing the vials into a tray freeze dryer (FTS Model FD-6-84C, Systems, Inc., Stone Ridge, NY) at a shelf temperature of −45°C and allowing the solutions/suspensions to freeze for 2 hours. Vacuum was applied and the shelf temperature was raised in 10°C increments every 6–10 hours, with a total cycle time of 50–60 hours. Final drying was at 35°C for 6 hours. After lyophilization the uncapped vials containing the amorphous powders were placed within various sealed % relative humidity (% R.H.) stability chambers at 35°C. The % R.H. was controlled by saturated salt solutions added to the stability chambers.

Moisture Analyses

After ≈5 days, a sample of lyophilized powder was removed from each relative humidity chamber and dissolved in 500 μl DMF which was previously dried using 4Å molecular sieves (EM Science, Cherry Hill, NJ). The water content was measured by Karl Fischer titration (Metrohm 684 KF Coulometer, Brinkman Instruments, Inc., Westbury, NY). Each titration consisted of injecting 250 μl of either blank DMF or a DMF/human insulin solution into the titrator with constant stirring, with the automatic titration continuing until all the water had reacted. The amount of water within the lyophilized powders was calculated by subtracting the amount of water in blank DMF from that measured in the DMF/human insulin solution. The % water content was calculated by multiplying by 100 the ratio of mass of water found to the initial dry mass of insulin.

The Guggenheim-Anderson-deBoer (GAB) equation (Eqn. [1]), an extension of the Brunauer-Emmett-Teller (BET) equation (25) developed for nonhomogeneous sorbents (26) and successfully employed in previous studies to describe protein sorption isotherms over the full range of relative humidity (5), was utilized in describing the moisture uptake results obtained in this study

$$W = \frac{W_m C_g K(p/p_o)}{[1 - K(p/p_o)][1 - K(p/p_o) + C_g K(p/p_o)]} \quad (1)$$

W in the above equation is the mg of water absorbed per mg of dry solid at a particular atmospheric water activity (p/p_o, or % relative humidity/100). W_m, which is also a term in the BET equation where it is referred to as the value for BET monolayer coverage, is often taken to approximate the amount of water necessary to saturate highly active sorption sites. The constants C_g and K in the model are related to thermodynamic measures of sorption for strongly and weakly interacting sorption sites (6).

HPLC Methods

All analyses of insulin and its degradation products were carried out by reverse phase HPLC using a modular system described previously (22). HPLC analytical columns and guard columns were: Rainin Microsorb-MV™ C8 300Å 5μ (4.6 × 250 mm) (Woburn, MA), or Applied Biosystems Brownlee Spheri-5 C-18 5μ (4.6 × 220 mm)(San Jose, CA). Elutions were performed using mixtures or gradients of mobile phases consisting of (A) 0.01M $(NH_4)_2SO_4$ pH 2.2 by concentrated H_2SO_4 and (B) acetonitrile (Baxter Inc., Muskegon, MI) with 0.07% (v/v) trifluoroacetic acid, or (C) 95% 0.1M Na_2HPO_4 pH 7.2, 4.5% methanol, 0.5% tetrahydrofuran and (D) methanol (Baxter Inc., Muskegon, MI).

Three HPLC methods, all at 35°C, were employed. HPLC method #1, used to quantitate human insulin and it degradation products, employed the Microsorb column with UV detection at 214 nm, a flow rate of 1.6 ml/min and a mobile phase initially at 72.5%A and 27.5%B followed by a gradient after 1.0 min (27.5%B to 30% B at 0.18%/min; after 15 min, 30%B to 35%B at 0.70%/min). HPLC method #2, utilized to separate the proteolysis fragments after enzymatic digestion with *Staphylococcus aureus* protease, used the Microsorb column with UV detection at 214 nm, a flow rate of 1.6 ml/min and a mobile phase initially at 84.0%A and 16.0%B followed by a gradient after 1.0 min (16.0%B to 28.4%B at 0.75%/min; after 18 min, 28.4%B to 50%B at 2.2%/min). HPLC method #3, employed for amino acid analyses, used the Brownlee column, fluorescence detection with excitation at 340nm and emission at 455nm, a flow rate of 1.0 ml/min and a mobile phase initially at 90%C and 10%D followed by a gradient after 5.0 min (10%C to 27%C at 0.7%/min; after 30 min, 27%C to 40% C at 0.37%/min; after 65 min, 40%C to 50%C at 1.0%/min; after 75 min 50%C to 65%C at 1.5%/min; after 90 min 65%C to 90%C at 15%/min).

Identification of Degradation Products

Lyophilized powders exposed to various % R.H.'s at 35°C for time periods up to several weeks were removed from the stability chambers, reconstituted with 1.0 mL deionized water, and analyzed by HPLC method #1. [$Desamido_{A21}$] insulin and the [Asp_{A21}-Phe_{B1}] amide linked dimer degradation products were identified by HPLC co-elution of authentic samples prepared previously (22,24). An additional degradation product (the [Asp_{A21}-Gly_{A1}] dimer) observed to form at higher levels in lyophilized powders than in solution was also isolated and characterized, as described below.

The suspected [Asp_{A21}-Gly_{A1}] covalent dimer was isolated from 'pH' 4.0 lyophilized powders containing zinc free human insulin which had been stored at 35°C and 96% R.H. for 14 days. After this period of exposure the suspected dimer accounted for about 10% of the decomposition products (the other 90% was accounted for by [$desamido_{A21}$] insulin and [Asp_{A21}-Phe_{B1}] dimer). Using HPLC method #1, the unidentified peak was collected, dialyzed against water and lyophilized. The product was enzymatically cleaved using *Staphylococcus aureus* protease strain V8 type XVII-B (P2922; Sigma Chemical Company, St. Louis, MO), which cleaves specifically at the carboxy side of Glu residues, in a pH 7.2 TRIS buffer for 3 hours at 35°C, according to the method of Grau (27). After digestion, the HPLC elution time of the modified fragment containing a new amide linkage was identified by comparison with the digestion pattern of unmodified human insulin using HPLC method #2. The modified fragment was collected, the CH_3CN evaporated off, and the remaining solution analyzed by electrospray FAB-Mass Spec (Finnigan MAT 95 High Resolution GC/Mass Spectrometer (Bremen, Germany) supported by a Finnigan MAT ICIS II Operating System) to determine the molecular weight of the modified fragment.

The modified fragment was further analyzed by amino acid analysis using HPLC method #3, as described elsewhere (24).

Kinetic Methods

At various times after placing lyophilized samples in the controlled relative humidity chambers, samples were removed and either assayed immediately or stored desiccated at 5°C. Assays performed after dissolving the powder in 1.0 mL deionized water included determination of pH and quantitation of the concentrations of intact human insulin, [$desamido_{A21}$] insulin, and the two covalent amide linked [Asp_{A21}-Phe_{B1}] and [Asp_{A21}-Gly_{A1}] dimers using HPLC method #1. Due to the limited solubility of human insulin at pH 5.0 (≈0.20 mg/mL), the pH 5.0 powders were first suspended in 1.0 mL deionized water, the pH was recorded, and the solutions were then acidified by adding 5 μL of 1.0 M HCl to dissolve insulin for HPLC analysis.

The initial (i.e., within the first 10% decomposition) rates of formation of degradation products, d[products]/dt, in lyophilized powders were divided by the average insulin concentrations in the reconstituted solutions (≈1.0 mg/mL) over the time frame of each experiment (Eqn. [2]) to obtain apparent first-order rate constants (k_{obs}). This normalization procedure, which assumes that the solid-state reaction follows first-order kinetics, afforded values which could be compared directly to the first-order rate constants obtained in 1.0 mg/mL solutions.

$$k_{obs} = \frac{d[products]/dt}{[insulin]} \quad (2)$$

The concentrations of insulin remaining in lyophilized samples and the degradation products formed were also monitored well beyond the first 10% degradation. These data could not be described by simple first-order kinetics, necessitating consideration of alternative kinetic schemes described below (see Kinetic Models).

Solution kinetics experiments were performed over the same pH range by adding human insulin to deionized water at 1.0 mg/mL (0.2 mg/mL for pH 5.0 solutions due to solubility limitations) then adjusting the pH with 1.0 M HCl to the pH values of the lyophilized powders after reconstitution (pH 3.1, 3.3, 4.1, and 5.0). These solutions were placed in a 35°C oven and assayed by HPLC method #1 at various time points. Rate constants were determined by the method of initial rates.

Anhydride Trapping with Aniline

Kinetic studies were also conducted using lyophilized powders containing residual aniline for the purpose of trapping as insulin anilides any anhydride formed in the solid-state decomposition of insulin. This approach had previously been employed to establish the formation of a cyclic anhydride intermediate in the solution degradation of human insulin (22). Solutions containing 1.0 mg/mL zinc free human insulin and 0.010, 0.10, 1.0 and 2.0 mg/mL aniline (Aldrich Chemical Company, Milwaukee, WI) at pH 4.0 were lyophilized in the same manner as described previously. The resulting amorphous white cakes were stored at 35°C and exposed to either desiccant or 75% R.H. At various time intervals a powder was removed from the stability chamber, reconstituted with deionized water, the solution pH was measured and the solutions were analyzed by HPLC method #1 for the concentration of [desamido$_{A21}$] insulin, covalent dimers, and anilides of insulin. The preparation and characterization of the two anilides formed from insulin in acidic solutions containing aniline ([N$^{\delta 2}$-phenyl Asn$_{A21}$] human insulin and [N$^{\gamma 2}$-phenyl Asp$_{A21}$] human insulin), used as reference standards in the present study, have been described previously (22).

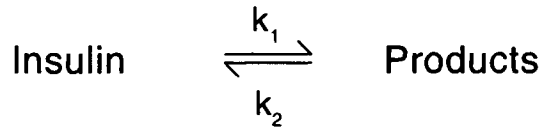
The residual aniline concentration in lyophilized powders was quantitated by dissolving samples in 2.0 mL 0.1M KOH and measuring the UV absorbance at 278 nm. The absorbance of a blank lyophilized powder with 1.0 mg/mL of insulin and no aniline was subtracted from the above measured absorbance. The difference in absorbance was converted to aniline concentration by using external standards of varying aniline concentration. A Perkin-Elmer Lambda 7 UV/VIS spectrophotometer (Norwalk, CT) was used for these absorbance measurements.

Kinetic Models

Rate Laws for Insulin Decomposition in the Solid State

Unlike the situation in solutions, semi-logarithmic plots of insulin remaining in the solid state versus time failed to follow simple first-order kinetics. Therefore, several alternative kinetic models were evaluated.

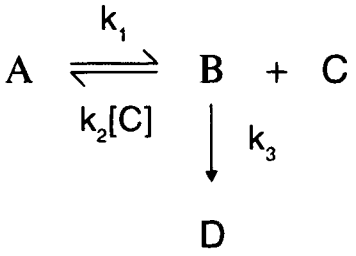
An empirical reversible first-order model depicted in Scheme I and Eqn. [3] was first employed to fit the experimental concentration vs. time profiles.



Scheme I.

$$[Products] = \frac{k_1[Insulin]_o}{k_1 + k_2}(1 - e^{-(k_1+k_2)t}) \quad (3)$$

A more conceptually realistic model consistent with the mechanism for the degradation of insulin established previously in solution (22) is that shown in Scheme II. This model assumes that the first-order formation of the cyclic anhydride intermediate (B) is reversible as a result of the second-order reaction of the anhydride with ammonia liberated in the forward reaction. The extent to which the reverse reaction becomes significant depends on whether or not ammonia builds up in the lypophilized solid as the reaction proceeds. The implicit equation used for computer fitting of the insulin concentration as a function of time is shown in Eqn. [4]:



Scheme II.

$$\left(\frac{A_o k_2}{k_1 k_3} + \frac{1}{k_1}\right)\ln\left(\frac{A_o}{A}\right) - \frac{k_2(A_o - A)}{k_1 k_3} = t \quad (4)$$

where A is insulin, B is the cyclic anhydride intermediate, C is ammonia, and D represents the combined degradation products.

Finally, an empirical model based on the assumption that insulin in the solid state exists in two non-interconverting conformational sub-states at initial concentrations A_{11} and A_{22} having different reactivities represented by the rate constants k_{11} and k_{22} leading to Eqn. [5] was evaluated:

$$[Insulin] = A_{11}e^{-k_{11}t} + A_{22}e^{-k_{22}t} \quad (5)$$

pH-rate profiles. The pH dependence of the degradation of insulin in solution was previously described by Eqn. [6] which attributes the reactivity of insulin to the fraction of the A21 terminal carboxyl group in the unionized form ($H^+/(H^+ + K_a)$) consistent with intramolecular catalysis, where K_a is the ionization constant for the A-21 carboxyl (22).

$$k_{obs} = \frac{k_{cat}H^+}{H^+ + K_a} \quad (6)$$

Rate constants obtained from the initial rates of product formation in lyophilized powders at various water contents and as a function of 'pH', defined as the pH of the formulation after reconstitution to a theoretical insulin concentration of 1.0 mg/mL, were also fitted to Eqn. [6].

Data Analysis

Computer fitting was performed using the appropriate equations and non-linear least squares regression analysis software (SCIENTIST, Micromath Inc., Salt Lake City, UT).

RESULTS

Physical Appearance of Lyophilized Powders

The lyophiles resulting from solutions initially at pH 2.0, 3.0 and 4.0 were all white cakes occupying a volume approximately equal to the original solution volume. In contrast, the pH 5.0 lyophiles were free-flowing powders rather than cakes. The solution pH after reconstituting the lyophilized powders in deionized water to a theoretical concentration of 1.0 mg/mL was in general slightly higher than the solution pH prior to lyophilization, presumably due to vaporization of some of the HCl during the freeze drying process. Thus, solutions initially at pH 2.0, 3.0, 4.0, and 5.0, respectively, yielded reconstituted solutions having pH values of 3.1, 3.3, 4.1, and 5.0. Lyophilized cakes exposed to desiccant retained 100% of their original volume with no evidence of collapse. Upon exposure to increasing % R.H. the cake volumes decreased. The rate of collapse was measured (not shown) for the pH 3.3 and pH 4.1 lyophilized powders and equilibrium heights were reached within 24 hours and maintained for the duration of the study. At 57% and 75% R.H., the pH 3.1, 3.3, and 4.1 products exhibited only partial collapse to 70–75% of their original volumes. At 96% R.H. the pH 3.1 and 3.3 cakes were fully collapsed, occupying <1% of their original volume, while the pH 4.1 cakes occupied 25% of their original volume. The pH 5.0 powders stored below 75% R.H. were free flowing, but exposure to 96% R.H. caused the powder to stick to the bottom of the vial.

Water Sorption of Lyophilized Insulin

The equilibrium water contents obtained for human insulin lyophilized powders at pH 3.1 and at pH 5.0 with and without NaCl are shown in Table 1. Water sorption isotherms of the pH 3.1 and 5.0 lyophilized powders (without NaCl), shown in Figure 1, indicate that as the powders were exposed to increasing % R.H. their equilibrium moisture content, W, increased. The solid curves in Figure 1 are nonlinear least squares best fits to the data using Eqn. [1] with a fitted value for W_m of 4.5% water for both the pH 3.1 and 5.0 powders, corresponding to 15 molecules of water for each molecule of human insulin. Above W_m, the pH 3.1 lyophilized powders are more hygroscopic than the pH 5.0 powders. The powders containing NaCl exhibited substantially greater hygroscopicity than powders without NaCl such that the pH 5.0 powders with NaCl had water contents as high as 990% after exposure to 96% R.H. at 35°C. (Table I).

Table I. Equilibrium Water Content [(mg water/mg dry protein) × 100] of Human Insulin pH 3.1 and 5.0 Lyophilized Powders at 35°C and Various % Relative Humidities

% Relative Humidity	pH 3.1[a]	pH 5.0[a]	pH 5.0[a,b]
0	2.7	3.1	12.0
11	4.3	5.1	13.2
32	8.6	6.6	13.7
54	10.4	9.0	20.0
75	13.8	14.0	34.4
83	21.9	16.9	234
96	52.5	33.8	990

[a] pH after reconstitution of the lyophilized powders with deionized water.

[b] Lyophilized powders contained ≈0.01 mmol NaCl per mg of insulin.

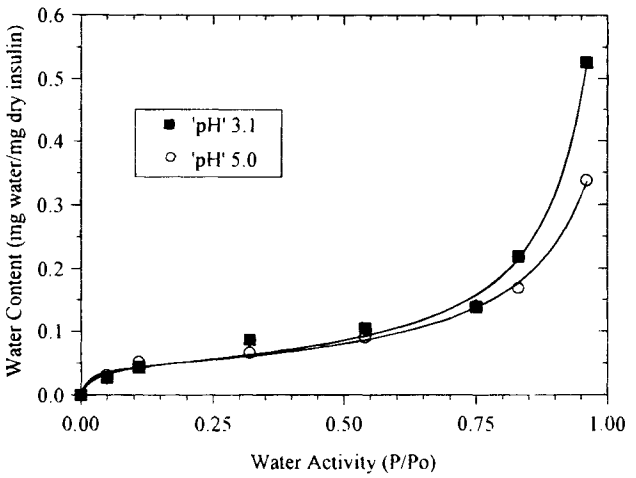


Fig. 1. Water sorption isotherms of pH 3.1 and 5.0 lyophilized human insulin at 35°C. The curves are non-linear least squares best fits using the GAB equation (Eqn. [1]).

Insulin Degradation Products

The major degradation products of human insulin in lyophilized powders prepared from solutions ranging from pH 2.0 to pH 5.0 as determined by HPLC co-elution with authentic standards were found to be the same as those formed in solution between pH 2–5 at 35°C, namely, [desamido$_{A21}$] human insulin and [Asp$_{A21}$-Phe$_{B1}$] insulin dimers (α- and β-linked). However, a significant product in lyophilized powders, but not in solution is the [Asp$_{A21}$-Gly$_{A1}$] insulin dimer. Identification of this amide-linked dimer was confirmed in a manner similar to that described for the [Asp$_{A21}$-Phe$_{B1}$] insulin dimer (24). Upon enzymatic digestion with *Staphylococcus aureus* protease human insulin is cleaved into 4 fragments while the [Asp$_{A21}$-Gly$_{B1}$] dimer is cleaved into 5 different fragments, 4 of which are identical to those formed from human insulin. The modified fragment containing the new amide linkage, shown in Figure 2, was determined to have a molecular weight of 1777 ± 3 by deconvolution of the electrospray FAB-mass spectrum (not shown) of the modified fragment. Of the 9 possible amide linked dimers resulting from the 3 amines of insulin (Lys$_{B29}$, the 2 N-terminal amines Phe$_{B1}$ and Gly$_{A1}$) and the three Asn's (Asn$_{A21}$, Asn$_{A18}$ and Asn$_{B3}$), the only possible covalent amide-linked dimers having this molecular weight are the [Asp$_{A21}$-Gly$_{A1}$] and the [Asp$_{A18}$-Gly$_{A1}$] dimers. Amino acid analysis of the modified fragment was also consistent with its assignment as either the [Asp$_{A21}$-Gly$_{A1}$] dimer or the [Asp$_{A18}$-Gly$_{A1}$] dimer, as these dimers contain the identical amino acids. However, since Asn$_{A21}$ is the reactive site in solution and lyophilized powders for deamidation and [Asp$_{A21}$-Phe$_{B1}$] dimer formation, it is reasonable to conclude that the dimer isolated is the [Asp$_{A21}$-Gly$_{A1}$] dimer.

Mechanism of Degradation

The method of aniline trapping was employed to determine whether or not a cyclic anhydride forms in the degradation of

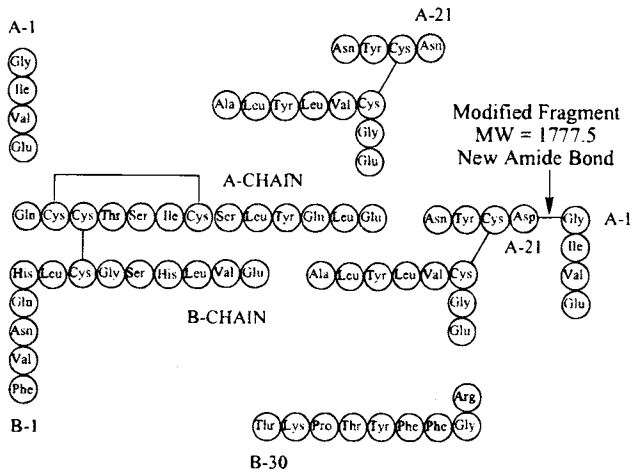


Fig. 2. [Asp_{A21}-Gly_{A1}] insulin dimer cleavage pattern resulting from *Staphylococcus aureus protease* digestion. The theoretical monoisotopic molecular weight of the modified fragment is 1777.5 amu.

lyophilized insulin as it does in solution between pH 2–5 (22,24). However, since aniline free base is a liquid at room temperature and at pH 4.0 exists as an equilibrium mixture of its neutral and protonated forms (pKa = 4.63), some of the free amine is vaporized during the freeze drying process, leaving a lower residual concentration of aniline in the lyophilized powders and a reduced pH after reconstitution of the lyophilized product compared to that of the solution prior to freeze drying (Table II). Lyophilized samples prepared from pH 4.0 solutions initially containing aniline at concentrations of 0.1, 1.0 and 2.0 mg/mL had 0.063, 0.14 and 0.16 mg/mL residual aniline (after reconstitution to 1.0 mg/mL insulin concentration) and pH values of 3.8, 3.7 and 3.6, respectively.

HPLC chromatograms of lyophilized powders containing residual aniline which were stored at 35°C in either 75% R.H. or in the presence of desiccant exhibited additional decomposition product peaks corresponding to the two anilide isomers forming at the A21 position, [$N^{\delta 2}$-phenyl Asn_{A21}] and [$N^{\gamma 2}$-phenyl Asn_{A21}] insulin (22). Lyophilized samples containing increased concentrations of residual aniline yielded increases in the fraction of overall products accounted for by the anilides (Figure 3), accompanied by decreases in the fractions of both [Asp_{A21}] insulin and covalent dimers formed.

The fact that increasing the concentration of residual aniline shifts the product distribution toward anilide formation at the expense of covalent dimer and deamidated products suggests competition for a common anhydride intermediate. If this shift occurs in the absence of an increase in the overall reaction rate as the residual aniline concentration increases, then the rate-limiting step must be the formation of the intermediate. To explore this possibility, the initial rates of insulin degradation in lyophilized powders with and without residual aniline were compared (Table II). The initial rates of product formation in lyophilized powders appear to be independent of aniline concentration after adjustment for pH differences and not significantly different from the rates in powders without aniline, suggesting that the direct reaction of aniline with the A21 position of insulin in the solid state under the conditions employed is negligible and that formation of the intermediate, a reaction which does not involve aniline, is rate-determining. It is possible, however, based on the above observations, that the cyclic anhydride intermediate accumulates in the solid state and that the degradation products observed on reconstitution are the result of the rapid reaction of the anhydride *after* reconstitution. To eliminate this as a possible explanation for the above results, a partially degraded pH 4.1 lyophilized powder having a water content of 3% and containing no residual aniline was reconstituted in a solution containing 0.2 M aniline at pH 4.0. No anilide formation was detected by HPLC, suggesting that the cyclic anhydride had already reacted with residual water and insulin in the lyophilized state, consistent with cyclic anhydride formation being the rate determining step.

Table II. Residual Aniline Concentrations, Reconstituted pH, Total Initial Rates of Insulin Loss, and Calculated Initial Rates of Insulin Loss in Lyophilized Powders at pH 4.0 and Various Initial Aniline Concentrations After Exposure to Desiccant and 35°C

% Relative Humidity	Initial Aniline (mg/ml)	Residual Aniline[a] (mg/ml)	pH After Reconstitution	Rate Constant[b] w/aniline (hr^{-1})	Rate Constant[c] w/o aniline (hr^{-1})
0	0.1	0.063	3.8	0.00024 ± 0.00003	0.00020
0	1.0	0.14	3.7	0.00026 ± 0.00004	0.00022
0	2.0	0.16	3.6	0.00027 ± 0.00004	0.00024

[a] After reconstitution; measured by UV absorbance at 278 nm.
[b] From initial rates of product formation ([desamido$_{A21}$] insulin, covalent dimers, and anilides) (see Eqn. [2]).
[c] Calculated using the pH after reconstitution and fitted parameters (pK_a = 3.85, and k_{cat} = 0.00037 hr^{-1}) from Table IV.

Kinetics of Insulin Decomposition as a Function of Water Content

The purities of the lyophilized insulin preparations were ≈98%, with [desamido$_{A21}$] insulin (≈1.5%) and covalent dimers (≈0.5%) the principal impurities. The total concentration of degradation products in lyophilized samples stored at

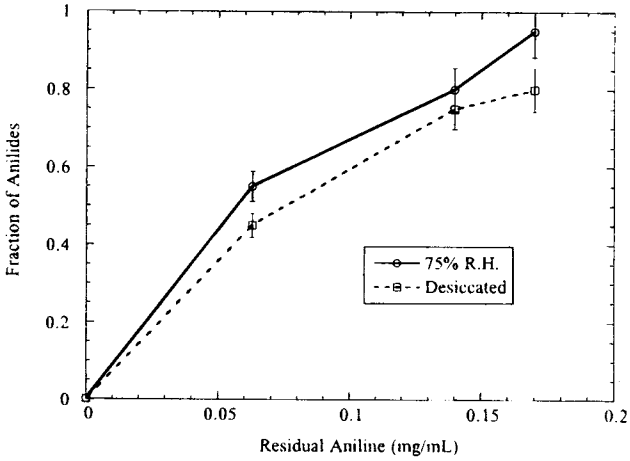


Fig. 3. Fraction of the overall products accounted for by anilides versus residual aniline concentration in pH 4.1 lyophilized powders at 35°C.

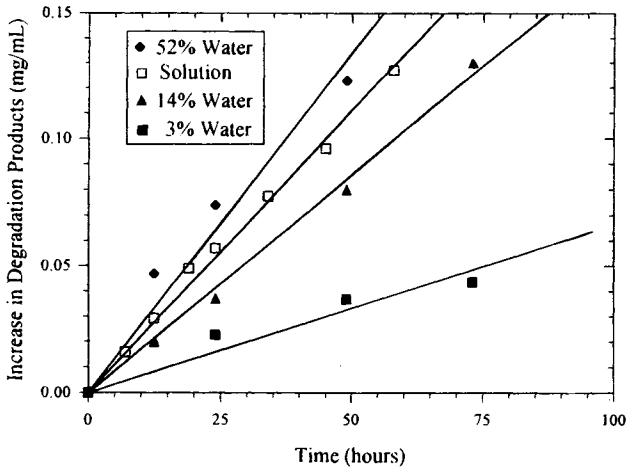


Fig. 4. Initial rate plots of the change in total concentration of degradation products at pH 3.3 and 35°C in 1.0 mg/mL insulin solution and lyophilized powders exposed to various % relative humidities.

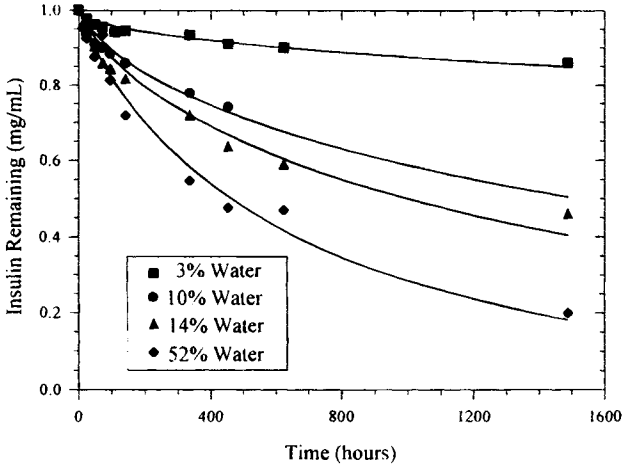


Fig. 6. Decrease in insulin concentration in pH 3.3 lyophilized powders (after reconstitution) exposed to various % relative humidities at 35°C. The curves are best fits to the data using Eqn. [4].

various % relative humidities increased with time and % water content, as demonstrated in Figure 4, which displays the initial rates of increase in degradation products (above the small amounts present at t_0) for pH 3.3 lyophilized powders (measured after reconstitution) and a solution at the same pH. These pH 3.3 initial rate plots are typical of all the pH values studied, in that the rates in hydrated lyophilized powders were generally within an order-of-magnitude of the rates in 1.0 mg/mL solutions at the same pH.

The rate constants obtained from the initial rates divided by insulin concentration (after reconstitution) as a function of water content in lyophilized powders are shown in Figure 5, where the "X's" designate the first-order rate constants in 1.0 mg/mL solutions at pH 3.3 and 4.0 and in 0.2 mg/mL solutions at pH 5.0. Insulin degradation rates increased steeply with water content at low levels of hydration, approaching apparent plateaus at water contents >20%. The rate constants in the plateau region were comparable to those in solution. At very high water contents (>50%), the initial decomposition rates were generally slightly faster than the rates in solution (see, for example, Figure 4 (upper curve)).

The kinetics of insulin decomposition in solution were shown to exhibit apparent first-order behavior over at least one half-life and first-order rate constants obtained from initial rates of product formation were identical to those obtained by monitoring insulin disappearance (22). Though not clearly evident in the initial rate plots in Figure 4, increases in degradation product concentration with time generally displayed deviations from linearity in the lyophilized powder data much earlier than would have been expected from simple first-order behavior. This may be seen more clearly in Figures 6 & 7, which depict the insulin remaining and the degradation products formed, respectively, in pH 3.3 lyophilized formulations versus time up to 62 days. The product concentration profiles in Figure 7 clearly show a leveling-off of product formation rates at longer time points. Several kinetic models, described in Schemes I & II and Eqns. [3]–[5], were found to provide reasonable fits to

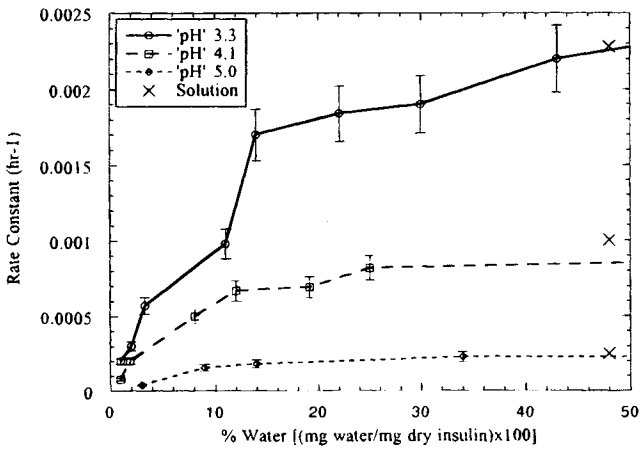


Fig. 5. Rate constants of 1.0 mg/mL insulin lyophilized powders at pH 3.3, 4.1 and 5.0 at 35°C as a function of water content. The X's designate the rate constants in solutions at pH 3.3 (1.0 mg/mL), pH 4.1 (1.0 mg/mL) and pH 5.0 (0.2 mg/mL).

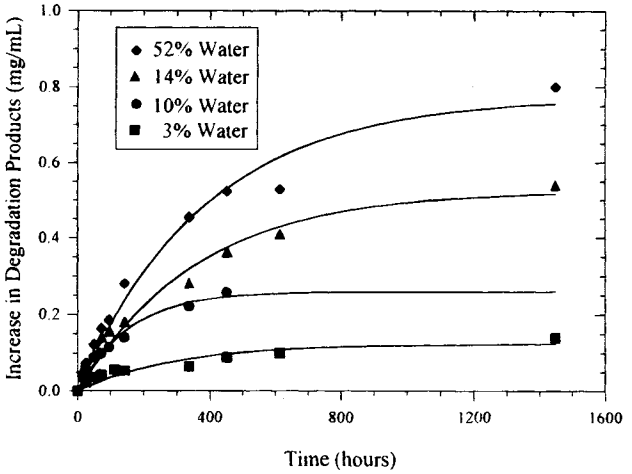


Fig. 7. Increase in total concentration of degradation products in pH 3.3 lyophilized insulin powders (after reconstitution) exposed to various % relative humidities at 35°C. The curves are best fits of the data using Eqn. [3].

the concentration-time profiles and thus it was not possible to unambiguously establish the most suitable kinetic scheme. For example, the solid curves passing through the insulin concentration-time data in Figure 6 were generated by fitting Eqn. [3] to the experimental points while the solid curves shown in Figure 7 were generated by fitting Eqn. [4] to the data. Eqn. [5] provided slightly better fits to all of the data but the degree of improvement was not sufficient to warrant inclusion of an additional adjustable parameter. Therefore, the simplest 2-parameter empirical model (Scheme I & Eqn. [3]) was used to generate the apparent forward and reverse first-order rate constants, k_1 and k_2, which are displayed in Table III. An inspection of the forward first-order rate constants obtained from the application of this empirical model shows that they agree reasonably well with the apparent first-order rate constants generated from initial rate data. While the k_1 values increased with increasing water content in the solid state, approaching the solution rate constant at high relative humidity, the k_2 values decreased substantially with increasing water content.

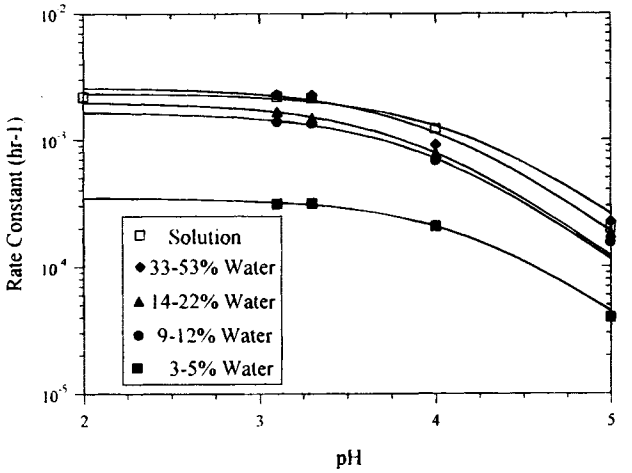


Fig. 8. pH-rate profiles for insulin solutions and lyophilized powders exposed to various % relative humidities at 35°C. The curves are best fits of the data using Eqn. [6].

pH-rate Profiles

Although the pH in the solid state is not well defined, the pH of the protein solution after reconstitution reflects the extent of ionization of the protein in both the solution and in the solid state, as the normal pK_a order of the acidic and basic functional groups within a protein are restored to approximately their solution values at very low levels of water content, between 0–8% (28,29). Reconstituted solution pH values were therefore employed in examining the pH dependence of insulin degradation in the solid state. The pH-rate profiles for solutions and lyophilized powders varying in water content are shown in Figure 8, where the curves are non-linear least squares best fits to the data using Eqn. [6] which mathematically accounts for the reactivity in terms of the fraction of the C-terminal A-21 carboxylic acid in the unionized (i.e., protonated form). The fitted parameters, k_{cat} and pK_a, are given in Table IV.

Remarkably, the pH-rate profiles (Fig. 8) generated from solutions and lyophilized powders have identical shapes, further supporting the conclusion that the mechanism of degradation is the same in solution and in lyophilized powders. The shapes of the pH-rate profiles are indicative of intramolecular catalysis, since the rates plateau at pH values below the pK_a of the terminal A21 carboxyl acid. The apparent pK_a for the A21 carboxyl was found to be the same in both solution and the solid state.

Also noteworthy in Figure 8 is the fact that the initial degradation kinetics in lyophilized powders are comparable to the rates in solution throughout the pH range at water contents >30%. At lower water contents, even under desiccated conditions (3–5% water), the rate of insulin decomposition in the solid state is slowed by less than a factor of 10 in comparison to that in solution.

Table III. Comparison of k_{obs} Values from Initial Rates of Product Formation with Reversible Rate Constants Obtained from Fitting Eqn. [3] to Compelete Concentration vs. Time Data in Lyophilized Powders and Solution at pH 3.3 and 35°C

% Relative Humidity	% Water	k_{obs}[a] (10^4 * hr^{-1})	k_1[b] (10^4 * hr^{-1})	k_2[b] (10^4 * hr^{-1})
Desiccated	3	2.5 ± 0.6	4.3 ± 3.3	48 ± 17
57%	10	14 ± 0.3	14 ± 0.3	30 ± 6
75%	14	17 ± 0.3	17 ± 7	13 ± 5
96%	52	26 ± 0.4	19 ± 2	5.9 ± 2.9
Solution	NA[c]	22 ± 0.4	NA	NA

[a] k_{obs} obtained from application of Eqn. [2] to initial rate data.
[b] Parameters obtained from application of Eqn. [3] to complete concentration vs. time profiles.
[c] Not applicable.

DISCUSSION

Water Sorption Isotherms

The sigmoid shaped water sorption isotherms of human insulin shown in Figure 1 exhibit characteristics which are typical of proteins (5,6). Two regions on these isotherms often serve as focal points. The first is the shoulder at a water content of ≈4.6%, corresponding to the value of W_m in the GAB (or BET) equation (Eqn. [1]). The second is the region in which a sharp rise in water uptake is observed, evident above a water content of ≈15–20%. Oksanen and Zografi have shown for polymer/water systems that the point on the isotherm where the amount of absorbed water increases significantly corresponds to the water content (W_g) which is suffi-

Table IV. Fitted Parameters (Eqn. [6]) for pH-Rate Profiles in Lyophilized Powders (pH 3–5) and Solution (pH 2–5) at 35°C

Medium	k_{cat}[a] (10^4 × hr^{-1})	pK_a[a]
Lyophilized powder, Desiccated	3.7 ± 1.2	3.85 ± 0.16
Lyophilized powder, 57% R.H.	13 ± 2	4.04 ± 0.14
Lyophiliized powder, 75% R.H.	15 ± 1	4.07 ± 0.14
Lyophilized powder, 96% R.H.	23 ± 1	4.13 ± 0.10
Solution	22 ± 1	3.92 ± 0.09

[a] Parameters obtained from fitting Eqn. [6] to k_{obs} values obtained from initial rates.

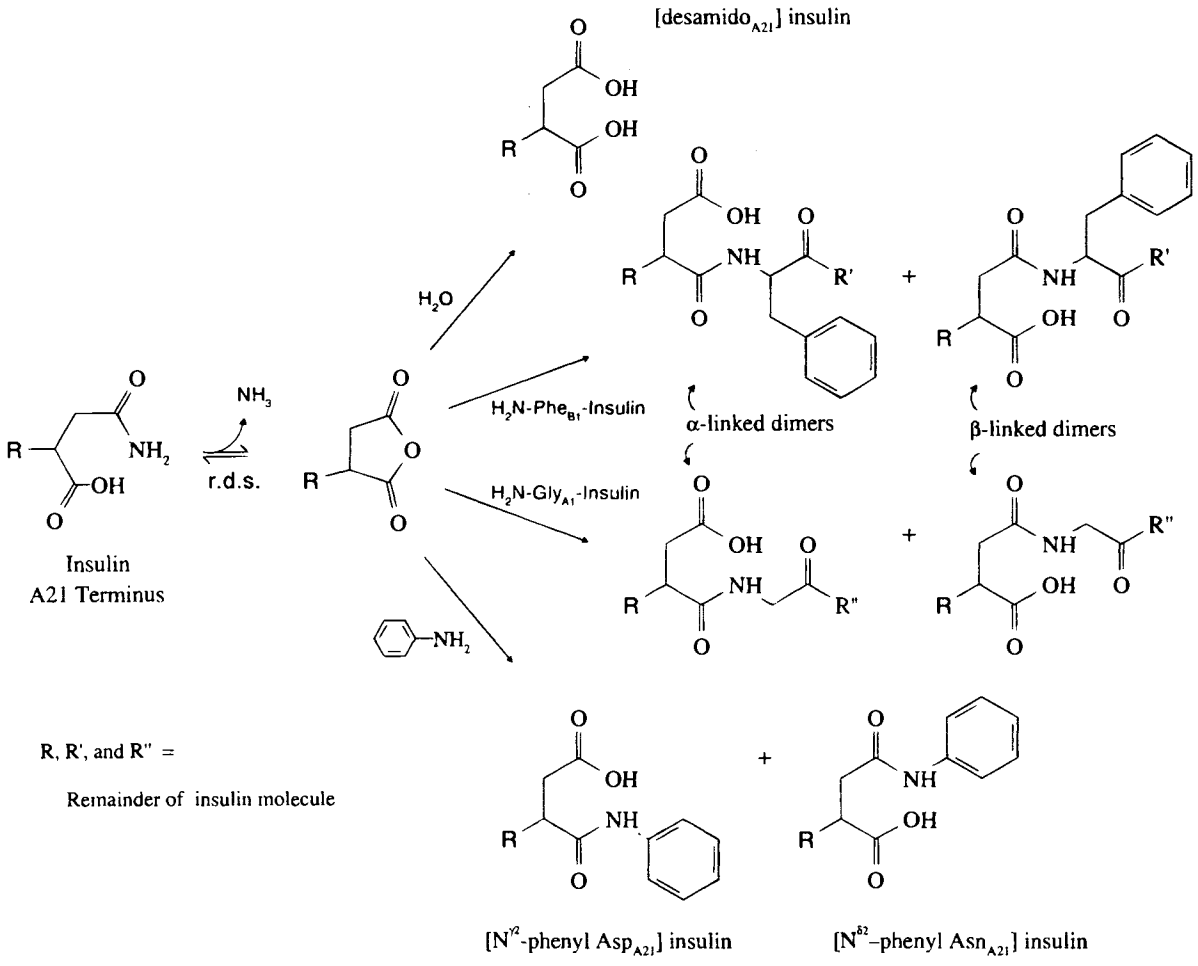
cient to lower the glass transition temperature of the solid to that of its environment (30,31). The complete collapse of the pH 3.1 and the partial collapse of the pH 4.1 lyophilized powders at 96% R.H. are consistent with the location of W_g and a possible glass transition between 75% R.H. (13% H_2O) and 96% R.H. (≈35% H_2O). Above W_g, the system is transformed into a rubbery state with greatly increased molecular motion. In this region of the isotherm, the diffusion coefficient of water approaches (but does not reach) that in bulk water (31). Enzymatic activity, which may involve cooperative changes in conformation over extended distances, also becomes significant above W_g (17).

It is clear, though, that molecular mobility is also significant in the glassy state of amorphous solids well below W_g (19), and even at water contents below W_m marked changes in local molecular motions can be observed with increases in water content (31). Adsorption models such as the BET or GAB equations which depict water as being "tightly bound" below full monolayer coverage at W_m and "loosely bound" above W_m seem inadequate to account for such observations. A conceptually more useful view, as pointed out in several recent publications (11,32,33), is that water dissolves in the disordered solid state, acting as a plasticizer to increase the free volume of the solid. In this context, increases in free volume accompanying the uptake of water would initially favor local conformational fluctuations and small molecular motion and only at relatively higher water contents would larger molecules (e.g., proteins) become mobilized.

Mechanism of Insulin Degradation in the Solid State

The mechanism of insulin degradation in lyophilized amorphous solids between pH 3–5, depicted in Scheme III, is similar to that in solution. The first-step, which is rate-determining, involves intramolecular nucleophilic attack of the C-terminal Asn_{A21} carboxylic acid onto the side-chain amide carbonyl resulting in the formation of a reactive cyclic anhydride intermediate which subsequently undergoes nucleophilic attack by one of several potential nucleophiles. The intermediate may react with water leading to [desamido$_{A21}$] insulin, or with the N-terminal Phe_{B1} or Gly_{A1} amino group of another molecule of human insulin to form the [desamido$_{A21}$-Phe_{B1}] or the [desamido$_{A21}$-Gly_{A1}] α- and β-linked dimers, respectively. In lyophilized powders containing residual aniline as a competing nucleophile, the anhydride intermediate reacts with aniline to form anilide products.

Several lines of evidence support the mechanism proposed in Scheme III. First, the pH-rate profiles generated in lyophi-



Scheme III.

lized powders are mathematically consistent with intramolecular catalysis by the protonated carboxyl terminus at the A21 position. The shapes of these profiles in the solid state parallel those in solution, with an apparent pK_a value in lyophilized powders of ≈4.0, compared with a kinetic pK_a found in solution (this study) of 3.9. It is probably coincidental that the pK_a values estimated from the solid-state kinetics agree so closely with the solution value. Previous studies in these laboratories have indicated that, in solution, the pK_a of the Asn_{A21} residue shifts from ≈4.1 in the insulin monomer to ≈3.3 in the self-associated dimer. The latter value agrees well with an estimated pK_a of 3.2 for the Asn_{A21} group in the dimeric Asp_{B9} mutant of human insulin determined from two-dimensional NMR titration studies (34), and may be rationalized by the formation of a salt bridge between the carboxylate group of Asn_{A21} and the guanidinium group of Arg_{B22} in the dimer. In solution at 1.0 mg/mL insulin exists in both monomeric and dimeric forms (23), so a kinetic value between the pK_a values for the monomer and dimer is reasonable.

A second observation consistent with the postulated mechanism is the similarity in degradation products formed in solution and in the lyophilized state both in the presence and absence of residual aniline. The formation of both deamidated insulin and covalent dimers in the solid state and the formation of anilides with a concurrent reduction in the fractions of both [desamido$_{A21}$] insulin and covalent dimers in the presence of aniline suggests competition for a common intermediate. In solution, the first-step was clearly established to be rate-limiting by examining the dependence of the overall decomposition rate on aniline concentration. This step appears to be rate-limiting in the solid state as well, as judged by the fact that the overall decomposition rate did not depend significantly on the residual concentration of aniline remaining in lyophilized samples over an aniline concentration range which resulted in a substantial shift in the product distribution profile.

In accordance with the proposed mechanism, the absence of a simple first-order dependence of the kinetics of insulin disappearance or product appearance in the solid state on insulin concentration was also shown to be rationalizable assuming a complex kinetic scheme (Scheme II) in which possibly the ammonia expelled in the first step reacts with the cyclic anhydride intermediate in a bimolecular reaction to regenerate insulin. A significant contribution of the reverse reaction of the cyclic anhydride with ammonia might not be expected in solution because the generated ammonia is free to diffuse throughout the solution, and would not attain a high local concentration in the vicinity of the cyclic anhydride intermediate. A similar type of first-order reversible kinetic treatment was also used to the fit data for the disappearance of model hexapeptides in pH 3.5–8.0 lyophilized powders with 0.3–2.7% water which underwent deamidation at Asn or isomerization at Asp via a cyclic imide intermediate (21). Further, these hexapeptides were shown to undergo the same reactions in solution (with different product distributions) but the solution degradation followed irreversible first-order kinetics. Further studies demonstrating the build-up of ammonia in the solid state and its back-reaction with the cyclic anhydride intermediate are necessary to confirm this speculative argument, however, as other models described in Eqn's. [3] & [5] could also fit the data.

The kinetics of insulin degradation in lyophilized powders are determined by the first step, unimolecular rate-limiting intramolecular cyclization, which presumably is dependent on conformational flexibility, whereas the product distribution is determined by the second step, bimolecular partitioning of the cyclic anhydride intermediate, and presumably is dependent on water mobility in deamidation and protein mobility in covalent dimerization.

The most obvious difference between the reaction pathway for human insulin in solution and in lyophilized powders is the formation of the [desamido$_{A21}$-Gly$_{A1}$] dimer in lyophilized powders to a significantly greater extent than in solution. There are 3 amino groups in human insulin which may participate as nucleophiles in reacting with a cyclic anhydride: the 2 N-terminal amino groups Phe_{B1} and Gly_{A1}, and the internal Lys_{B29}. The pK_a values of these groups are: $Phe_{B1} < 7.0$; $Gly_{A1} \approx 8.0$; and $Lys_{B29} > 9.0$ (35,36). The formation of [Asp_{A21}-Phe_{B1}] dimer as the major dimer, [Asp_{A21}-Gly_{A1}] dimer being a minor product, and the absence of formation of the [Asp_{A21}-Lys_{B29}] dimer are consistent with the order of the pK_a values of the 3 amino groups in solution. Lower pK_a values favor nucleophilicity at pH values less than the pK_a, as they lead to a higher concentration of the deprotonated nucleophilic species at a given pH value. The greater extent of formation of [Asp_{A21}-Gly_{A1}] dimer in the solid state is not fully understood. One possibility is that within the random organization of the amorphous glassy solid state, there may be a certain fraction of favorably oriented nearest neighbors in which the cyclic anhydride intermediate of one molecule is in close contact with the Gly_{A1} N-terminal free amino group of a second molecule thereby facilitating the formation of the [Asp_{A21}-Gly_{A1}] dimer even though the translational freedom necessary for protein-protein reactions may be limited. This explanation has been invoked previously to account for aggregate formation of human growth hormone in the glassy solid state (37).

Kinetics as a Function of Water Content

Knowledge that the rate-limiting step in the degradation of insulin in the solid state in the pH 3–5 range involves an intramolecular (i.e., unimolecular) reaction between two functional groups separated by only a few angstroms provides the opportunity to consider the influence of viscosity and molecular mobility within amorphous solids, as altered by water uptake, on the kinetics of unimolecular reactions occurring in the solid state in more general terms. Intramolecular reaction mechanisms quite similar to that under investigation herein are quite common in protein decomposition in both solution and solid states. Deamidation at internal Asn residues under slightly acidic and neutral pH conditions, for example, involves intramolecular nucleophilic attack of a peptide amide nitrogen on a side-chain amide residue to form a cyclic imide intermediate which further reacts with water to form Asp and iso-Asp deamidated analogs (38,39). Unlike the cyclic anhydride in the present study, cyclic imide intermediates have been shown to accumulate in the solid state under certain conditions (21). Internal peptide bond hydrolysis at the nitrogen side of Ser (4,40) and the carboxy side of Asp (1,41,42) are also hypothesized to occur via an intramolecular cyclic intermediate. Diketopiperazine formation at the N-terminus of proteins with Pro or Gly at the second amino acid occurs via intramolecular cyclization with the expulsion of a new protein minus the 2 N-terminal amino acids (4,43).

Qualitatively, the role of water as a plasticizer is evident in the increases in reactivity of insulin with increasing water content, observed in Figures 4–8. Unexpected, perhaps, is the finding (5) that even at relatively low levels of hydration (<5% H_2O, below W_m) insulin degrades at a rate which is only 10 times slower than in solution and at a rate comparable to that in solution above 10–15% H_2O (near W_g). In general, glass transition temperatures for proteins are relatively high at low moisture contents (44). Thus, the 35°C storage temperatures of the present study should be well below T_g for samples containing <5% H_2O. Substantial reactivity well below T_g would seem to conflict with the notion that all motion relevant to stability correlates with the reciprocal of viscosity, as embodied in the Williams-Landel-Ferry (WLF) relationship, which relates viscosity to T-T_g, where T_g is the glass transition temperature (45,46). WLF theory suggests that motion essentially ceases below the glass transition temperature, and indeed, some studies have shown dramatic decreases in reactivity as a glass transition was approached (9). However, several investigators have observed that at least some molecular motions do not cease in the glassy state (3,19,31). For example, in NMR studies of correlation times for several types of motion in a polymer system, Kohlhammer *et al.* observed (3,47) that chain reorientation was strongly coupled to the glass transition while side-chain aromatic ring flips were nearly completely decoupled from the glass transition. Similarly, diffusion of small molecules (e.g., water) in polymers appears to be decoupled from the glass transition (31). Pikal concluded from these observations that mobility involving motion on a larger scale appears to correlate best with viscosity (3).

The reacting functional groups of insulin in the formation of the cyclic anhydride intermediate, a carboxylic acid and an amide carbonyl, are already in close proximity to each other and require only short-range conformational flexibility to react. Cyclic anhydride formation may also require one or more proton transfers in order to proceed to completion, but even a small amount of water localized near the cyclic anhydride intermediate may have sufficient short-range mobility to act as a medium for proton transfer during the cyclization step. The conclusion that the rate-limiting step is intramolecular cyclization, even in relatively dry powders, implies that there is enough localized conformational flexibility to form the cyclic anhydride intermediate and enough available water to react with the cyclic anhydride once it is formed. At 4.5% water (i.e., W_m) there are 15 molecules of water for each molecule of insulin, most of which may be localized in close proximity to polar residues. Thus, it seems reasonable that even at low water contents there will be sufficient water located near the C-terminal Asn_{A21} for the reactions in question to proceed to completion.

One implication of these studies is that significant stabilization of proteins which undergo decomposition via unimolecular processes involving residues in close proximity may require very low moisture contents (i.e., highly immobilized systems) since local conformational motions are the last to be restricted in the glassy state. On the other hand, bimolecular reactions which require mobilization of at least one of the reacting molecules in order for the reactants to obtain the proper orientation and sufficient energy to react may be affected more dramatically in the solid state even at higher moisture contents. A subsequent paper in this series will address the influence of moisture and pH on the bimolecular partitioning of the cyclic anhydride intermediate of human insulin to [$desamido_{A21}$] insulin and covalent dimers in lyophilized powders.

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