Articles

Acid Stabilization of Insulin

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ABSTRACT: The effect of pH on the conformational stability of insulin was studied. Surprisingly, the Gibbs free energy of unfolding increased ~30% by acidification. pH titration of insulin's conformational stability is described by a transition involving a single proton with an apparent pK_a of 7.0. The acid stabilization of insulin's conformation was attributed to the protonation of histidine at position 5 on the B-chain (H^{B5}) as determined by ^{1}H-NMR of the histidines, selective amino acid alteration, and enthalpies of ionization. Further acidification (at least to pH 2) does not decrease the free energy of unfolding. A conformational change in the tertiary structure, as indicated by the near-UV circular dichroism spectrum, accompanies this change in stability. We propose that this acid stabilization of insulin is physiologically important in maintaining insulin stability in the acid environment of the secretory/storage granules of the β-cell of the pancreatic islets of Langerhans.

Insulin is a small polypeptide hormone that is integrally involved in the homeostasis of blood glucose levels. The physiological responses evoked by insulin are the direct result of an interaction between the molecule and specific cell surface receptors. Elucidation of the three-dimensional structure of insulin and its relationship to function have been the subject of intense research over the past several decades. Insulin has served as a paradigm in the development of the principles governing the relationship between the structural and the functional properties of proteins.

Insulin is a two-chain globular protein in which the A-chain (21 residues) contains an intrachain disulfide bond and is connected to the B-chain (30 residues) through two interchain disulfide bonds. X-ray crystallographic (Baker et al., 1988) and solution NMR[1] studies (Kline & Justice, 1990; Hua et al., 1991) indicate that the core structure of insulin involves the packing of three helical segments spanning residues A^{1-9} and A^{12-19} of the A-chain and B^{9-19} of the B-chain. However, the insulin structure is highly dynamic, with the N- and C-terminal segments of the B-chain having the greatest flexibility (Krueger et al., 1987; Chothia et al., 1983; Derewenda et al., 1991). One conformation of insulin (R-state) has the central helix of the B-chain elongated to the N-terminus, whereas in another conformation (T-state) this segment is in an extended conformation (Brader & Dunn, 1991). Fluorescence-detected equilibrium denaturation of an insulin analog containing a unique tryptophan probe in the C-terminal segment of the B-chain revealed the preferential unfolding of this portion of the molecule compared to the remaining structure (Bryant et al., 1992). The 2Zn hexamer crystal form is composed of three dimers that are made up of two insulin molecules (monomer 1 and monomer 2) that are conformationally different (Baker et al., 1988). Thus, multiple conformations of insulin have been identified by several biophysical techniques. The flexibility of the insulin conformation has been implicated as an essential property for receptor binding (Derewenda et al., 1991).

The biosynthesis of insulin occurs within the β-cells of the pancreatic islets of Langerhans and constitutes an archetypical pathway for the regulated processing of secretory proteins (Halban, 1991). Subsequent to translation within the cytoplasm, the single-chain preproinsulin is transported into the endoplasmic reticulum where the signal sequence is removed and the resulting proinsulin molecule is packaged into secretory/storage granules (Halban, 1991). The proteolytic conversion of proinsulin to insulin occurs concomitant with the acidification of the secretory granule by an ATP-dependent proton pump and the removal of the clathrin coat from the exterior of the granule. Once processed, insulin may remain within the acidified granules for several days (Halban, 1991). Thus, during the course of insulin biosynthesis, storage, secretion, circulation, and its ultimate interaction with the insulin-specific receptor, the molecule is subjected to a wide variety of environmental conditions, including variations in pH.

The difference between the free energies of the native and denatured states of globular proteins is relatively small (5–20 kcal/mol). The spontaneous folding of a protein is thermodynamically favored by hydrogen-bonding, hydrophobic, van der Waals, and electrostatic interactions that are formed in the native state. Variations of the solution pH can significantly alter the electrostatic interactions by altering the charge present on those amino acid residues with ionizable side chains. Human insulin contains a total of 16 ionizable groups (2 N-terminal amino groups, 2 C-terminal carboxyl groups, 4 Glu residues, 2 His residues, 4 Tyr residues, 1 Lys residue, and 1 Arg residue) (Brown et al., 1955). The overall net

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[1] Abbreviations: Gdn-HCl, guanidine hydrochloride; BHI, biosynthetic human insulin (pertinent amino acids or amino acid replacements are denoted by the single-letter amino acid code followed in superscript by its location in the A- or B-chain and sequence position; ornithine is denoted by the three-letter code Orn); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; MRE, mean residue ellipticity; NMR, nuclear magnetic resonance; MES, 4-morpholineethanesulfonate.

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charge of insulin is important to its solubility. From pH 4.5 to pH 6.5, insulin is insoluble due to isoelectric precipitation (pI = 5.5), and as a result, little is known concerning any conformational changes within this pH range. Below pH 4.5, hexamer formation is disrupted due to protonation of H^{B10} and loss of the metal ion coordination site, but no other acid-induced conformational changes have been reported.

In contrast, the propensity of insulin to self-associate to dimers and higher order aggregates under a variety of solution conditions has been thoroughly described. In the presence of divalent metal ions, insulin specifically aggregates to a hexamer with two metal atoms coordinated to the unprotonated imidazole moieties of H^{B10} (Adams et al., 1969; Bradbury et al., 1981). Detailed atomic information concerning insulin's self-association has come from X-ray crystallography of a variety of crystal forms (Bentley et al., 1976; Chothia et al., 1983; Baker et al., 1988; Derewenda et al., 1989; Badger et al., 1991). The structural integrity of insulin within the secretory granules is thought to be enhanced by the formation of quasi-crystalline hexamers induced by the binding of both zinc and calcium metal ions. The structural elements involved in stabilizing the conformation of insulin as it is exposed to these varying conditions and the contribution of electrostatic interactions, other than those involved in the coordination of metal ions, are only marginally understood.

We have reported on the equilibrium denaturation of insulin under solution conditions that maintain a monomeric state (Brems et al., 1990). During denaturation studies we observed that denaturant concentrations of 2–4 M Gdn-HCl or urea did not induce denaturation and were sufficient to preserve solubility throughout the isoelectric region. The use of nondenaturing concentrations of chaotropic reagents provides a new opportunity to explore the solution conformation of insulin in a previously inaccessible pH range. The goal of our current investigation was to obtain a more thorough understanding of the role that electrostatic interactions play in stabilizing the structure of insulin. Here, we describe studies involving changes in the conformation and thermodynamic stability of human insulin as a function of solution pH.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure Gdn-HCl and urea were purchased from ICN Biochemicals. HEPES buffer was of enzyme grade and was obtained from Fischer Scientific. Anhydrous absolute ethyl alcohol (200 proof) was supplied from Quantum Chemicals. N^{α}-Acetyl-L-histidine methyl ester was of research grade and was purchased from Serva. All other chemicals were of analytical grade or higher.

Synthesis and Isolation of BHI, D^{B10} Insulin, Orn^{B5} Insulin, and N-Carbamoylated Derivatives. BHI was obtained from Eli Lilly and Co. A human insulin analog in which H^{B10} was substituted with an aspartic acid residue was prepared by site-directed mutagenesis and purified from a culture of bacteria harboring the appropriate proinsulin expression vector. The identity of D^{B10} insulin was confirmed by fast atom bombardment mass spectrometry and *Staphylococcus aureus* protease V8 peptide mapping. The purity of BHI and D^{B10} insulin was >98% and 93%, respectively, as determined by analytical reversed-phase chromatography and contained <0.5% high molecular weight contaminants according to size exclusion HPLC. A human insulin analog in which H^{B5} was replaced with ornithine (Orn^{B5}) was prepared by semisynthesis. The insulin B-chain, incorporating the His → Orn replacement, was synthesized with an ABI Model 430A peptide synthesizer using Boc-protected amino acid derivatives. Synthetic insulin B-chain was combined with recombinant insulin A-chain by the method of Chance et al. (1981). The identity of Orn^{B5} insulin was confirmed by amino acid analysis and fast atom bombardment mass spectrometry. The purity of Orn^{B5} insulin was 92% as determined by analytical reversed-phase chromatography. N-Carbamoyl-A^1 insulin and N,N-dicarbamoyl-A^1,B^1 insulin were prepared by reacting 0.5 g of human insulin with 0.25 g of NaCN in 100 mL of 10 mM Tris-HCl, 1 mM EDTA, and 7.5 M urea, pH 8.5, for 24 h at 25 °C. N-Carbamoyl-B^1 insulin and N,N-dicarbamoyl-A^1,B^1 insulin were separated by chromatography on a preparative C-18 reversed-phase HPLC column.

Equilibrium Denaturation Measurements. Gdn-HCl-induced equilibrium denaturation experiments were performed as described previously (Brems et al., 1990). Samples of varying Gdn-HCl concentrations were prepared by mixing varying ratios of a denaturant stock solution and a standard buffer composed of 50 mM Tris-HCl, 25 mM MES, 25 mM acetate, 1 mM EDTA, and 20% (v/v) ethanol, pH 8.0. Denaturant concentrations were determined by differential refractive index measurements using a Bausch & Lomb refractometer. The addition of 20% ethanol reduces the number of species present under native conditions such that a two-state model can be applied to the analysis of equilibrium denaturation experiments (Brems et al., 1990).

Circular dichroism measurements were performed using an Aviv 62D spectropolarimeter at a protein concentration of 0.1 mg/mL in a cylindrical cell with a path length of 0.500 cm at 23 °C. The Gdn-HCl-induced unfolding transition was followed by observing the mean residue ellipticity (MRE) at 224 nm. The raw data were then used in the calculation of the Gibbs free energy of unfolding using the linear extrapolation method of Pace et al. (1987).

Near-UV spectra were collected at 23 °C using a protein concentration of 1.00 mg/mL. Far-UV spectra were recorded using a 0.020-cm cell and a protein concentration of 0.1 mg/mL. Results are reported as mean residue ellipticity having units of deg·cm^2·dmol^{-1} and were calculated using a mean residue weight of 115 Da. Solvent conditions were the standard buffer solutions used in the equilibrium denaturation experiments. Concentrations of human insulin, D^{B10} insulin, and Orn^{B5} insulin were determined by UV absorbance spectroscopy using an extinction coefficient of 1.05 mg mL^{-1} cm^{-1} at 276 nm (Frank & Veros, 1968).

Acid Stabilization of the Insulin Conformation. The Gibbs free energy of unfolding for human insulin was determined at different pH values from the results of individual Gdn-HCl-induced equilibrium denaturation experiments at the specific pH. The pH of each denaturation experiment was determined by averaging the individual values taken from samples proximate to the midpoint of the transition, using an Orion Model 811 pH meter. Typically, the pH of each sample was found to deviate less than ±0.05 pH unit from the average value determined within the transition region of a given equilibrium denaturation experiment, irrespective of the target pH.

pH-Induced Near-UV CD Conformational Transitions. The circular dichroism intensity at 253 nm was recorded for a sample composed of 1 mM human insulin or an analog, 50 mM Tris-HCl, 25 mM MES, 25 mM acetate, 1 mM EDTA, 20% (v/v) ethanol, and 3 M urea in a 0.200-cm cuvette at 23 °C. Each point in a given titration experiment represents the average intensity observed over a 60-s period at a sampling rate of 1 point/s. The pH of a 5-mL working stock solution was adjusted by the addition of microliter quantities of

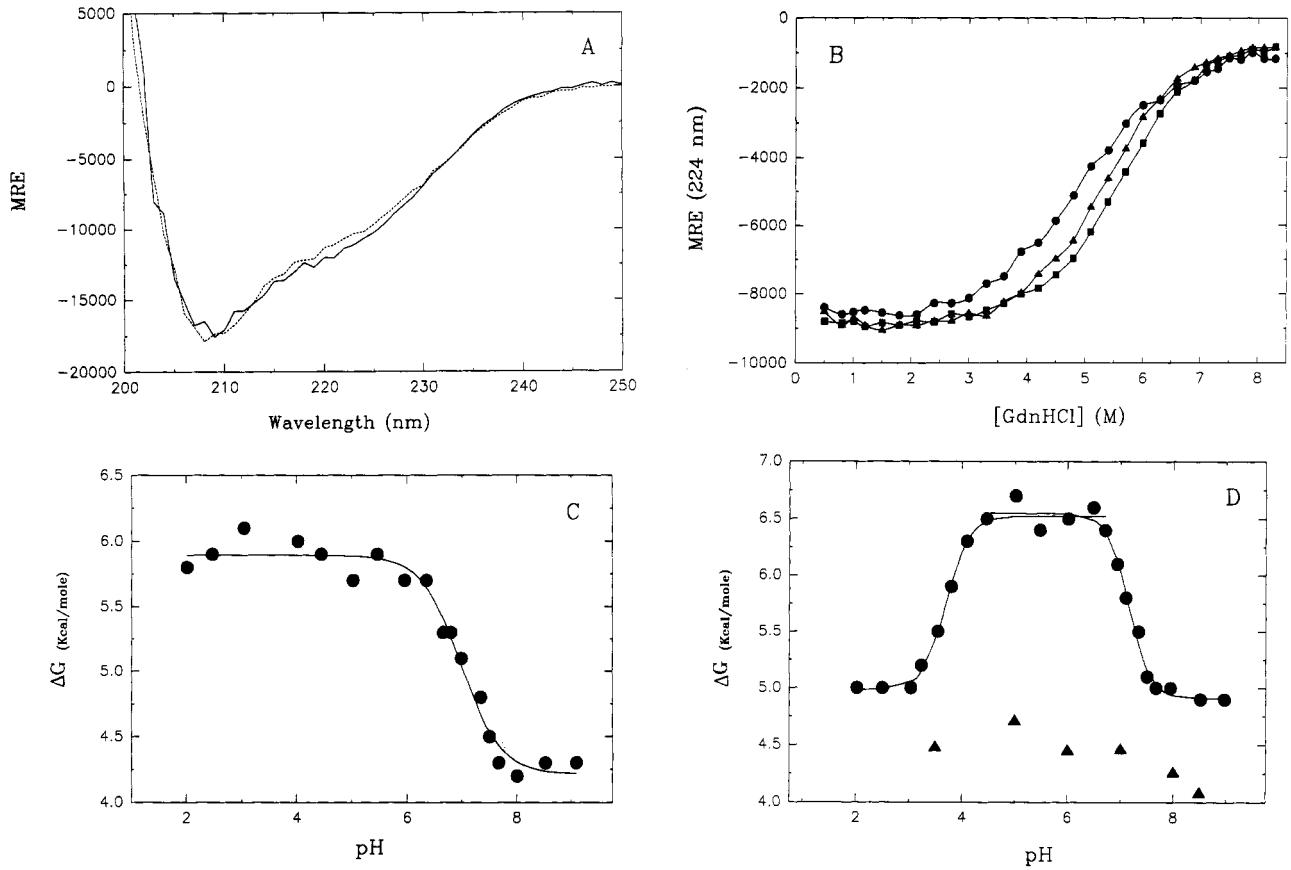

FIGURE 1: Acid stabilization of insulin's secondary structure. (A) Far-UV CD spectra of insulin at pH 2 (dashed line) and pH 8 (solid line). (B) Gdn-HCl-induced equilibrium denaturation transitions for insulin at pH 3 (■), pH 7 (▲), and pH 9 (●). The lines in (B) simply connect the data points. (C) pH titration of the free energy of unfolding of insulin. (D) pH titration of the free energy of unfolding of D^{B10} insulin (●) and Orn^{B5} insulin (▲). The lines in (C) and (D) represent the best fit to the data according to a least-squares analysis using a modified three-parameter Henderson–Hasselbach equation as described in the Results section.

concentrated HCl or NaOH. The relative effect of the pH adjustments on protein concentration and the resulting calculated MRE value were negligible. The addition of 3 M urea maintained solubility of BHI and the various insulin analogs throughout the isoelectric region. The presence of 3 M urea in this system was found to have little effect on the far-UV CD spectrum and was found to be within the native state baseline of the urea-induced equilibrium denaturation curves (data not shown). Significant changes were noted in the near-UV CD spectrum of BHI upon the addition of 3 M urea. However, the observed differences were found to be insignificant at 253 nm.

Enthalpy of Ionization Measurements. The enthalpy of unfolding was determined from the relationship

$$\ln K = -H/RT + S/R$$

where H is enthalpy, T is temperature in kelvin, S is entropy, and R is the gas law constant. The equilibrium constant K was determined from equilibrium denaturation experiments using the relationship

$$G = -RT \ln K$$

where G is the Gibbs free energy of unfolding. Enthalpy of ionization was determined by conducting the equilibrium denaturation experiments at pH 4 and 8 at varying temperatures. For temperature equilibration, an external circulating water bath was used to maintain samples at the desired temperature. Within the temperature range of 25 and 39 °C, ln K was a linear function of $1/T$. The slope of the straight line at each pH equals $-H°/R$.

Equilibrium Ultracentrifugation. Analytical sedimentation studies were obtained using a Spinco Model E analytical ultracentrifuge with a photoelectric scanning optical system operated at 22 °C. Samples were dissolved in the same solvent as used for the pH-induced conformational transitions or in the same solvent minus the urea and ethanol. The weight average molecular weight was calculated assuming ideal solution conditions.

NMR Methods. The ^{1}H-NMR spectra were recorded at 299.949 MHz at 25 °C on a Varian Unity 300 spectrometer using a spectral width of 5047.3 Hz, 64K time–domain data points, and 90° pulse excitation. Solutions (1 mM) of insulin, D^{B10} insulin, or N^{α}-acetyl-L-histidine methyl ester were prepared in 0.05 M deuterated Tris, 3.0 M urea-d_4, 0.025 M sodium acetate-d_3, and 20% (v/v) ethanol-d_4. Labile protons were exchanged with deuterium, and the pH was adjusted by the addition of 35% DCl and 40% NaOD as necessary. The pH was measured in the deuterated solution by using a glass electrode calibrated with standard buffers without applying correction for deuterium isotope effects (Bundi & Wuthrich, 1979). Chemical shifts were determined using the deconvolution routine in the standard Varian software. Chemical shifts are referenced to sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ (TSP-d_4).

RESULTS

Acid Stabilization of the Insulin Conformation. Far-UV CD spectra indicated that the secondary structure of metal-free insulin does not differ significantly at neutral and acidic

pH (Figure 1A). The effect of pH on the Gdn-HCl-induced equilibrium denaturation transitions is illustrated in Figure 1B. The Gibbs free energy of unfolding for BHI at various pH's was determined, and the results are illustrated in Figure 1C. The data exhibit a single titration transition between pH 5.5 and pH 8.0 with an increase in free energy of unfolding ($\Delta\Delta G$ = 1.8 kcal/mol) upon acidification. This transition was fit to the following modified three-parameter Henderson–Hasselbach equation employing a nonlinear least-squares analysis:

$$y = \{A + (1/10^{pH})^n/[(1/10^{pH})^n + (1/10^{pK})^n]\}(AH - A)$$

where y is the observed value, A represents the baseline value for the unprotonated species, AH is the baseline value for the protonated species, and n is the Hill coefficient for the titration. The calculated fit to the data is indicated by the solid line in Figure 1C and yields an apparent pK_a of 7.0 with the titration of a single proton.

To explore the contribution of H^{B10} and H^{B5} to the acid-induced stabilization of insulin, the pH-induced changes in the Gibbs free energy of unfolding for D^{B10} insulin and Orn^{B5} insulin were measured, respectively. The pH dependence on the Gibbs free energy of unfolding for D^{B10} insulin is described by two independent titration curves. The first transition occurs in the neutral pH range with a pK_a ~7.2 and is similar ($\Delta\Delta G$ = 1.6 kcal/mol) to that observed for BHI. A second transition is observed in the acid pH range with an apparent pK_a of ~3.7 and a decrease in the relative stability from 6.5 to 5.0 kcal/mol ($\Delta\Delta G$ = 1.5) as the pH is further reduced. In contrast to BHI and D^{B10} insulin, the unfolding of Orn^{B5} insulin is not dependent on pH. These results show that protonation of H^{B5} for insulin causes the acid-pH enhancement of the Gibbs free energy of unfolding.

pH-Induced Conformational Properties in Nondenaturing Solutions. The secondary structure of insulin in nondenaturing solutions is not perturbed by acidification (Figure 1A); however, the near-UV spectrum at neutral pH is different from that observed at acidic pH (Figure 2A). Spectral changes in the near-UV reflect changes in the ellipticity of the aromatic amino acid side chains or disulfide bonds. Spectral changes in the near-UV could result from conformational changes in the tertiary stucture or in changes of the quaternary structure. The effect of pH on the CD signal at 253 nm is shown in Figure 2B. A sigmoidal transition with a shape and apparent pK_a (~7.0) similar to the pH-induced changes in Gibbs free energy (Figure 1C) was obtained. These similarities in the pH-induced transitions detected by the near-UV and the Gibbs free energy of unfolding strongly suggest a common origin. The advantage of the near-UV-detected pH-induced transition is that it requires less material because the transition can be obtained from a single sample whose pH is adjusted consecutively compared to a Gdn-HCl-induced transition that requires a separate sample for each point in the transition.

To investigate the proton responsible for the pH-induced transition, the near-UV-detected pH transition was determined for *N*-carbamoyl-A^1B^1 insulin (the N-terminal amines are blocked and incapable of ionization in the pH range of interest) and for Orn^{B5} insulin. Figure 2B shows that the pH-induced near-UV CD-detected transition exists for *N*-carbamoyl-A^1B^1 insulin and is identical to insulin. However, the transition is absent for Orn^{B5} insulin, indicating that protonation of H^{B5} is responsible for the pH titration effect.

pK_a of Histidine B^5 and B^{10} As Determined by ^{1}H-NMR. The pK_a of the histidines was determined to elucidate the origin of the ionizable species responsible for the observed

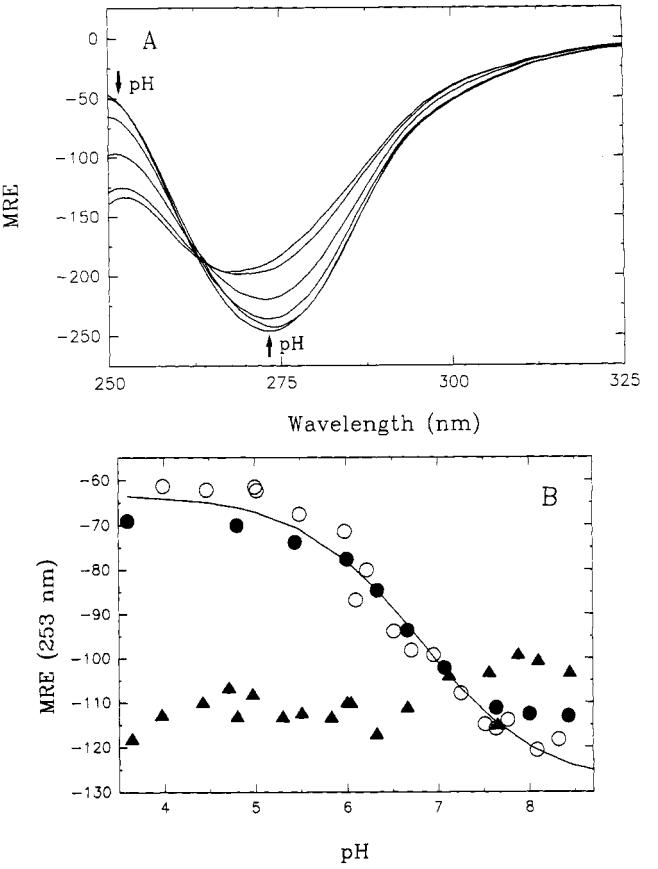

FIGURE 2: Acid-induced conformational change as detected by near-UV CD. (A) Near-UV CD spectra of insulin at pH 8.48, pH 8.08, pH 6.95, pH 5.98, pH 5.0, and pH 4.47. The pH's of the spectra are indicated by the direction of the arrows in the figure. (B) pH titration of the near-UV CD at 253 nm for BHI (○), *N*-carbamoyl-A^1B^1 insulin (●), and Orn^{B5} insulin (▲). The line is the best fit curve as described in Figure 1 to both insulin plus carbamoylated insulin.

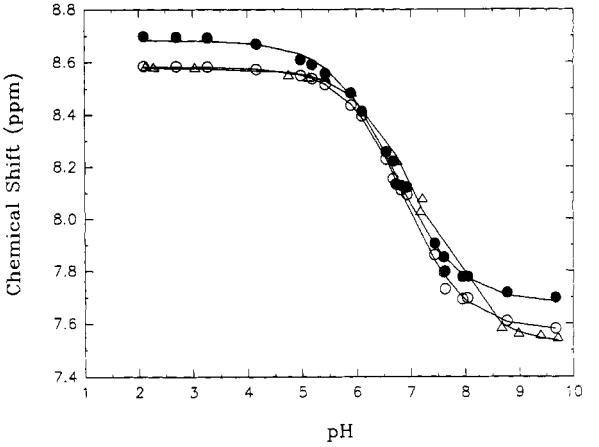

FIGURE 3: pH titration of the ^{1}H-NMR chemical shifts of the C2 protons of H^{B10} (●), H^{B5} (○) of insulin, and H^{B5} (△) of D^{B10} insulin. The lines represent the best fit curves to each of the data sets as described in Figure 1.

pH-induced transition. Figure 3 illustrates the pH dependency for the chemical shifts of the C2 protons of H^{B5} and H^{B10} (Cheshnovsky et al., 1983). H^{B5} of human insulin has a pK_a of 6.9 and H^{B10} of 6.7 in a nondenaturing and solubilizing solvent (3 M urea). H^{B5} of D^{B10} insulin has a pK_a of 7.2. ^{1}H-NMR titration of N^{α}-acetyl-histidine methyl ester, in the same solvent as used for insulin, shows a pK_a of 6.3 in 3 M urea and 6.4 in the absence of urea. Therefore, the folded structure of insulin raises the pK_a of H^{B5} by ~0.8 pH unit (average of urea-corrected human insulin and D^{B10} insulin is

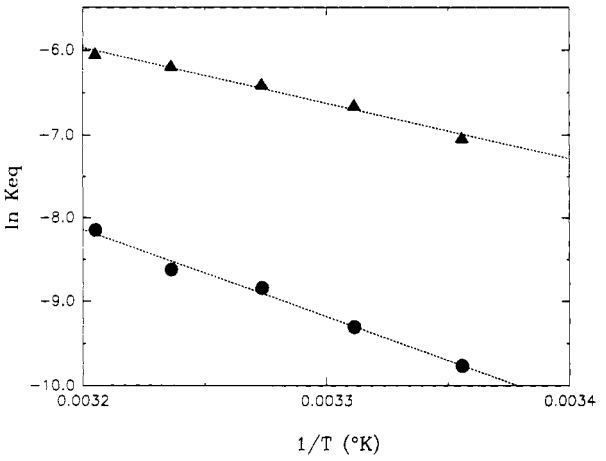

FIGURE 4: Enthalpy of ionization. The K_{eq} of unfolding was determined by Gdn-HCl-induced denaturation at pH 8 (▲) and pH 4 (●) as a function of temperature.

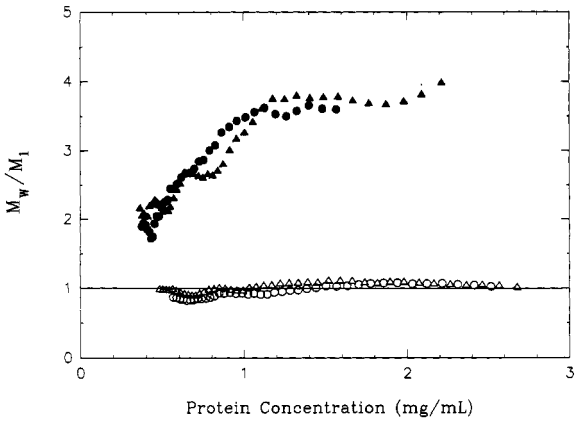

FIGURE 5: Equilibrium ultracentrifugation. (▲) is the equilibrium sedimentation results at pH 3.7 and (●) is those at pH 8 in aqueous buffer. (△) is the equilibrium sedimentation results at pH 3.7 and (○) is those at pH 8 in 3 M urea and 20% EtOH. M_w/M_1 represents the weight average molecular weight obtained from ultracentrifugation divided by the molecular weight of the insulin monomer.

7.2) and of H^{B10} by 0.4 pH unit. Either histidine has a pK_a that is within experimental error of the pK_a observed for the transitions of Figures 1 and 2.

Enthalpy of Ionization. Ionizable amino acid side chains have distinct enthalpies of ionization. For example, carboxyl acids have ΔH's of <2 kcal/mol, ammonium ions have ΔH's of 10–12 kcal/mol, and imidazoles have ΔH's of 7.7 kcal/mol (Nozaki et al., 1957). The results in Figure 4 show that the activation energy for unfolding at pH 8 is 13.0 kcal/mol and at pH 4 is 20.7 kcal/mol. The enthalpy of ionization for the pH-induced transition (Figure 1C) represents the difference in activation energy between pH 8 and pH 4, which is 7.7 kcal/mol. Therefore, the enthalpy of ionization establishes that the ionization of a histidine is responsible for the pH-induced transitions observed in Figures 1 and 2.

The Acid Stabilization Is Not Due to Self-Association. To determine if changes in association state contribute to the observed pH-induced transitions, the ultracentrifugation of insulin was studied under the solution conditions used in Figures 1 and 2. The equilibrium ultracentrifugation results of insulin at pH 3.7 and 8 in the presence and absence of 3 M urea and 20% ethanol are illustrated in Figure 5. The ultracentrifugation results show that, in 3 M urea and 20% ethanol, insulin is monomeric at pH 3.7 or 8, and in aqueous solvent (absence of urea and ethanol), self-association occurs with no difference between pH 3.7 and pH 8. We conclude that the pH-induced change observed in Figures 1 and 2 is a tertiary conformational change that is not accompanied by any significant changes in the secondary or quaternary structures.

DISCUSSION

Acid Stabilization. The unusual finding in this study is that the conformation of insulin is stabilized by acid. Acid stabilization of insulin is due to the protonation of a single site with a pK_a of ~7.0. Further acidification (to at least pH 2) does not result in decreased conformational stability. As the net charge on insulin is increased by increasing acidity, the charge repulsion would be expected to destabilize the folded state because the charge density of the folded molecule is greater than for the unfolded state. Hence, acid destabilizes native proteins (Privalov, 1979; Dill, 1990). To our knowledge, insulin is the only protein that possesses an increased free energy of unfolding as a result of acidification to low pH. Acid stabilization of insulin is accompanied by a change in the tertiary structure as observed by an alteration in the near-UV CD spectrum but with no detectable perturbations in the secondary or quaternary structures as determined by far-UV CD and equilibrium ultracentrifugation, respectively. The near-UV CD spectral change that results from acidification is due to a change in the optical activity of one or more of the tyrosine(s) and/or the disulfide bond(s) (Strickland & Mercola, 1976). Protonation of the other acid groups (except for protonation of the responsible site for acid stabilization) must not perturb any stability requiring interactions. This is consistent with the measured solvent exposure of the acid side chains of the insulin monomer as derived from X-ray crystallography of the 2Zn porcine hexamer (Baker et al., 1988). Using the method of Lee and Richards (1971) for determining accessible surface area, all the acid-titratable groups of Zn-free monomeric insulin (E^{A4}, E^{A17}, H^{B5}, H^{B10}, E^{B13}, E^{B21}, A-chain and B-chain C-terminal carboxyls) have >90 Å^2 area of contact with solvent (personal observation).

Protonation of H^{B5} Is Responsible for Acid Stabilization. The possible protonation sites responsible for acid stabilization are H^{B5}, H^{B10}, either N-terminal amino or C-terminal carboxyl, and any one of the four glutamic acid side chains. To determine the protonation site responsible for acid stabilization, the following experiments were conducted: proton NMR pH titration of the C2 histidine residues, enthalpy of ionization, and selective amino acid substitution or modification. The C2 histidine titration was carried out in a nondenaturing solvent (3 M urea, 20% ethanol) that maintained the solubility of the monomeric state of insulin throughout the entire pH range. Control experiments with N^{α}-acetyl-L-histidine methyl ester show a pK_a of 6.3 in the same solvent as used for the insulin titration (containing 3 M urea) and a pK_a of 6.4 in the absence of urea. Therefore, 3 M urea depresses the pK_a of histidine by 0.1 pH unit. Figure 3 shows that, in a solvent containing 3 M urea, H^{B5} has a measured pK_a of 6.9 in insulin and 7.2 in D^{B10} insulin, and H^{B10} has a measured pK_a of 6.7. After correction for the small effect of 3 M urea on the pK_a of C2 protons of histidine, H^{B5} has an intrinsic pK_a of 7.2 (average of human insulin and D^{B10} insulin), and H^{B10} has an intrinsic pK_a of 6.8. Either of the histidine side chains has perturbed pK_a's and is consistent within experimental error to the pK_a observed for the acid stabilization. According to the near-UV CD in Figure 2B, insulin blocked by carbamoylation at the two N-termini, *N*-carbamoyl-A^1B^1 insulin, is still capable of demonstrating the tertiary conformational change that accompanies the acid stabilization. Since the α-amino groups

of *N*-carbamoyl-A^1B^1 insulin are blocked and incapable of titration in the pH range investigated, we conclude that titration of the N-terminal amines is not responsible for the acid stabilization. Titration of Orn^{B5} insulin resulted in no change in the near-UV CD (Figure 2B). This indicates that protonation of H^{B5} is responsible for the pH-dependent change observed for insulin in the near-UV CD. The different types of side chains (i.e., amino, carboxyl, imidazole) are known to have distinct enthalpies of ionization (Nozaki et al., 1957). Figure 4 illustrates that the activation energy for unfolding at pH 8 is 13.0 kcal/mol and at pH 4 is 20.7 kcal/mol. The difference in the activation energies of unfolding between pH 8 and pH 4 is 7.7 kcal/mol and represents the enthalpy of ionization for the acid stabilization of insulin. The enthalpy of ionization for the acid stabilization is consistent with an imidazole ionization. This supports the conclusions of the NMR titration studies and the near-UV titration studies of *N*-carbamoyl-A^1B^1 insulin.

To further confirm which histidine is responsible for the acid stabilization, the pH titration of the free energy of unfolding for D^{B10} insulin and Orn^{B5} insulin was determined. Figure 1D demonstrates that D^{B10} insulin still undergoes an increase in the free energy of unfolding by acidification with a pK_a of ~7. Under more acid conditions, D^{B10} insulin demonstrates another titration event (pK_a 3.7) that decreases the free energy of unfolding to the level comparable at pH 8. We suggest that the decrease in free energy of unfolding observed in the more acidic region, pK_a 3.7 for D^{B10} insulin, is due to the protonation of the aspartic acid at B^{10}, which is normally a histidine in wild-type insulin. Figure 1D further shows that Orn^{B5} insulin lacks the pH-dependent change in free energy of unfolding. We conclude that H^{B5} is the protonation site responsible for the observed acid stabilization in the conformation of insulin. The pK_a of H^{B5} is raised 0.8 pH unit compared to N^{α}-acetyl-L-histidine methyl ester in the same solvent. This implies that the protonated form of imidazole is stabilized by ~1.1 kcal/mol relative to the uncharged form (Van Holde, 1985). Gdn-HCl-induced equilibrium denaturation shows that the acid form is stabilized by 1.7 kcal/mol for human insulin and 1.6 kcal/mol for D^{B10} insulin. These values of the acid stabilization obtained by NMR titration and equilibrium denaturation are consistent within the experimental errors of the different methods.

Careful examination of the X-ray structure of 2Zn porcine insulin hexamer (Baker et al., 1988) reveals that H^{B5} is within possible hydrogen bond distance of two main-chain carbonyls. Figure 6 is a computer-derived representation of the local environment of H^{B5} in molecule I and molecule II of the asymmetric dimer pair from the 2Zn structure of porcine insulin. The models were constructed using an Evans and Sutherland PS390 workstation running INSIGHT software from Biosym, Inc. H^{B5} is observed in two distinct conformations, each exhibiting differential hydrogen-bonding patterns. In molecule I, two hydrogen bonds through the $N\delta^1$ position are possible, one involving the main-chain carbonyl oxygen of residue C^{A7} at a distance of 2.0 Å and a second hydrogen bond to the main-chain carbonyl oxygen of S^{A9} at a distance of 2.5 Å (Figure 6). In contrast, only one possible hydrogen bond is seen in molecule II, involving the $N\epsilon^2$ hydrogen of H^{B5} and the main-chain carbonyl oxygen of C^{A7} at a distance of 1.5 Å (Figure 6). We suggest that the observed acid stabilization is due to the protonation of H^{B5} resulting in the formation of a new hydrogen bond to the carbonyl oxygens of C^{A7} and/or S^{A9}. The increased stabilization of 1.5 kcal/mol (average values obtained from equilibrium denaturation and NMR titration) agrees with the value expected for the contribution of a hydrogen bond (Dill, 1990).

An alternative explanation for the observed stabilization is a charge–helix dipole interaction (Scholtz & Baldwin, 1992). The charge–helix dipole interaction would occur between protonated H^{B5} and the partial charges on the non-hydrogen-bonded CO groups at the C-terminus of helix A^{1-9}, which are within van der Waals contact distance.

The histidine at B^5 was substituted in order to verify its role in the acid stabilization of insulin. Orn was chosen as the substituted amino acid at B^5 with the expectation that at neutral pH the protonated δ-amino group of Orn might substitute for the protonated δ^1 nitrogen of H^{B5} that exists for insulin at acid pH. In this manner the acid stabilization observed for insulin might be achieved at neutral pH with Orn^{B5} insulin. Figures 1D and 2B show that the ΔG of unfolding and the near-UV CD of Orn^{B5} insulin at all pH's tested are at the level obtained for insulin if H^{B5} is unprotonated (pH >7.5). Therefore, the expected achievement of increased free energy of unfolding at neutral pH by the Orn^{B5} insulin was not realized.

Physiological Relevance. The observed stabilization of insulin in acid may have important implications to in vivo and in vitro properties. According to a thermodynamic explanation for protein stability (McLendon & Radany, 1978), the native state of a protein is known to provide protection against degradation. Accordingly, the stability of a protein is governed by the equilibrium constant of unfolding

$$K_{eq} = U/N$$

where N = native and U = unfolded; for the reaction N ↔ U. Solvent conditions (such as pH changes) or amino acid changes that decrease K_{eq} are expected to increase the stability of a protein. In vivo, insulin is stored for varying lengths of time in an acid environment prior to secretion into the blood stream. The in vivo exposure of insulin to acid occurs as a result of the proteolytic conversion of proinsulin to insulin within the secretory granules. The biosynthesis of insulin occurs via the single-chain polypeptide preproinsulin, which is converted to proinsulin shortly after (or during) translocation into the lumen of the rough endoplasmic reticulum (Halban, 1991). Proinsulin is then transferred to the Golgi apparatus, where it is directed toward nascent secretory granules. Conversion of proinsulin to insulin and C-peptide arises within secretory granules and is dependent upon their acidification. The granules serve as the storage depot from which insulin can be secreted via a regulated pathway. Once processed, insulin may remain in the acidified secretory granule for several days before being released or degraded (Halban, 1991). Granule acidification occurs by an ATP-dependent proton pump, and in the β-cell, the mature granule has a pH ~5.5, which is more acidic than the immature granule (Hutton, 1982). The endopeptidases responsible for conversion of proinsulin to insulin show a narrow pH optimum of activity of 5.5 (Davidson et al., 1988). The acidification of the secretory granule milieu is a prerequisite for efficient conversion. The observed acid stabilization of insulin may be important in stabilizing insulin in the environment of the secretory granules that may be related to defective insulin production in certain disease states, including diabetes mellitus. H^{B5} is a conserved residue throughout the different species, with coypu (a South American rodent closely related to the guinea pig) being an exception with R^{B5} (Smith, 1972).

The in vitro storage stability of Zn-free insulin has recently been shown to depend on the conformational stability as predicted by the thermodynamic explanation of protein

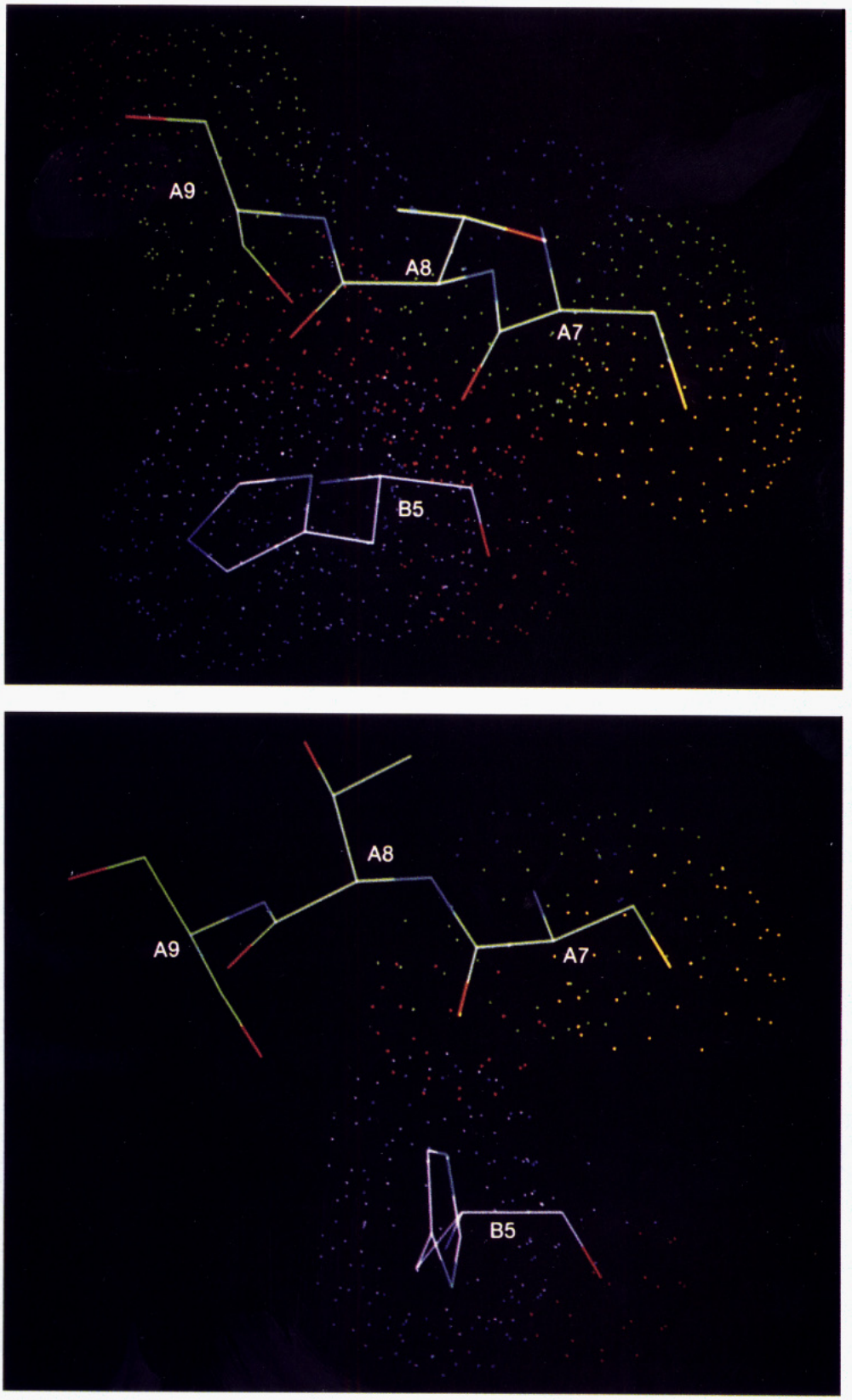

FIGURE 6: Putative hydrogen bonding of protonated H^{B5}. Computer representation of the local environment of H^{B5}. H^{B5} is observed in two distinct conformations, each exhibiting different hydrogen-bonding patterns. (A, top) In molecule I, two hydrogen bonds through the $N\delta^1$ position are possible. The first involves a contact with the carbonyl oxygen of C^{A7} at a distance of 2.0 Å while the second hydrogen bond is to the carbonyl oxygen of S^{A9} at a distance of 2.5 Å. (B, bottom) In contrast, only one hydrogen bond is seen in molecule II involving the $N\epsilon^2$ hydrogen which interacts with the carbonyl oxygen of C^{A7} at a distance of 1.5 Å. The α-carbon atoms are indicated by numbers for the sequence position, and the letters A and B signify the chain.

stability (Brems et al., 1992). Thus, the observed acid stabilization of insulin has important implications for the shelf-life storage of pharmaceutical formulations of insulin.

The acid stabilization of insulin may also have important consequences on the conformational flexibility of insulin. Protonation of H^{B5} and formation of the proposed hydrogen

bond may decrease the conformation flexibility of insulin. Recent studies suggest that the N-terminal and C-terminal regions of the B-chain are dynamic in nature and sample a large number of conformationally distinct "substate" structures (Krueger et al., 1987; Chothia et al., 1983; Derewenda et al., 1991). Current concepts concerning the nature of the interactions between insulin and the insulin receptor have begun to focus on the need for a conformational change and/or conformational flexibility within the insulin structure upon binding with the receptor (Derewenda et al., 1991). Thus, conformational heterogeneity within specific structural regions of insulin has very important consequences on the functional properties of the molecule. For example, the titration of H^{B5} may function as a switch between two conformational states: (1) the protonated form, which is less flexible but more stable under storage conditions; and (2) the unprotonated form, which is less stable but more amenable to receptor binding due to increased flexibility.

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