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Equilibrium Denaturation of Insulin and Proinsulin

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ABSTRACT: The guanidine hydrochloride induced equilibrium denaturation of insulin and proinsulin was studied by using near- and far-ultraviolet (UV) circular dichroism (CD). The denaturation transition of insulin is reversible, cooperative, symmetrical, and the same whether detected by near- or far-UV CD. These results are consistent with a two-state denaturation process without any appreciable equilibrium intermediates. Analysis of the insulin denaturation data yields a Gibbs free energy of unfolding of 4.5 ± 0.5 kcal/mol. Denaturation of proinsulin detected by near-UV CD appears to be the same as for insulin, but if detected by far-UV CD appears different. The far-UV CD results demonstrate a multiphasic transition with the connecting peptide portion unfolding at lower concentrations of denaturant. Similar studies with the isolated C-peptide show that its conformation and susceptibility to denaturation are independent of the rest of the proinsulin molecule. After the proinsulin denaturation results were adjusted for the connecting peptide contribution, a denaturation transition identical with that of insulin was obtained. These results show that for proinsulin, the connecting peptide segment is not a random coil; it is an autonomous folding unit, and the portion corresponding to insulin is identical with insulin in terms of conformational stability.

Insulin is a 2-chain (A and B) globular protein of 51 amino acids containing 1 intrachain and 2 interchain disulfide bonds (Brown et al., 1955). Extensive X-ray crystallographic studies of insulin have been carried out and have provided detailed information concerning its secondary, tertiary, and quaternary structures (Adam et al., 1969; Baker et al., 1988; Chang et al., 1986; Wang et al., 1982). Insulin's secondary structure is approximately 40% helical with helices spanning residues A1–A5 and A13–A19 of the A chain and B9–B19 of the B chain. An extended chain persists from B1–B8 and B21–B30 of the B chain. Several crystal forms have been studied by X-ray diffraction (Bentley et al., 1976; Dodson et al., 1978). Each crystal structure differs slightly, with the principle difference lying at the N- and the C-terminus of the B chain.

The solution behavior of insulin is known to be complex. The metal-free species exhibits a pH- and concentration-dependent association pattern consisting of monomer, dimer, tetramer, etc., all in dynamic equilibrium (Fredericq, 1956; Jeffrey & Coates, 1966; Pekar & Frank, 1972; Goldman & Carpenter, 1974; Jeffrey et al., 1976; Pocker & Biswas, 1981). Zn and other divalent metal ions induce the specific formation of an insulin hexamer (Fredericq, 1956; Cunningham et al., 1955). The association and crystallization behavior of insulin has been utilized by the pharmaceutical industry to effect changes in the time action of insulin formulations (Davidson, 1980). Highly aggregated or crystalline formulations are slower to dissociate and result in extended action. The active form is thought to be monomeric (Frank et al., 1972a). Insulin association has been studied by a wide range of spectral and physical techniques such as light scattering (Bohidar & Geissler, 1984), optical spectroscopy (Rupley et al., 1967; Pocker & Biswas, 1980; Goldman & Carpenter, 1974), ultracentrifugation (Jeffrey & Coates, 1966; Pekar & Frank, 1972; Goldman & Carpenter, 1974; Mark et al., 1987), and equilibrium dialysis (Grant et al., 1972; Goldman & Carpenter 1974). There are no dramatic changes in the insulin conformation upon dilution to the monomeric state, but small changes, of unknown significance, have been observed (Pocker & Biswas, 1980; Goldman & Carpenter, 1974).

Insulin is derived from a single-chain precursor, proinsulin (Steiner & Oyer, 1967). A 35 amino acid fragment designated as the connecting peptide is removed from the middle of proinsulin to form insulin (Steiner et al., 1971). The biological activity of proinsulin is only about 10–20% that of insulin (Peavy et al., 1985). The conformation of proinsulin has not been determined by X-ray diffraction, but optical spectroscopy studies support the contention that the insulin portion of the proinsulin molecule is in the same or very similar conformation

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to that of the insulin molecule (Frank et al., 1972a).

In this report, we have studied the guanidine hydrochloride (GdnHCl)[1] induced equilibrium denaturation of insulin and proinsulin. Providing that the disulfides remain intact, these protein molecules can be unfolded and refolded multiple times without hysteresis. We have used circular dichroism (both near- and far-ultraviolet) to follow the folding of insulin and proinsulin. The far-UV CD measures the integrity of the secondary structure while the near-UV CD reflects the environment of the aromatic and cystine amino acids. The near-UV CD is particularly sensitive to insulin association. This is thought to represent the change in environment around the tyrosines as a result of association (Ettinger & Timasheff, 1971). In order to study the equilibrium denaturation of insulin and proinsulin, the contribution of association must be carefully accounted for. This was achieved by including 20% ethanol, which (i) decreases the association such that insulin monomer can be studied at appropriate concentrations for spectroscopy, (ii) has little effect on intrinsic conformation, (iii) and does not alter the receptor binding capacity (Michael Weiss and Steven Shoelson, personal communication, Harvard Medical School).

Experimental Procedures

Materials. Insulin and proinsulin were obtained from Eli Lilly & Co. utilizing recombinant DNA technology (Goeddel et al., 1974a,b). Insulin was derived from proinsulin by limited proteolysis (Frank et al., 1981). These protein samples were greater than 98% pure on a protein basis and had less than 20 ppm of Zn or other divalent metal ions. C-Peptide was derived from proinsulin using the combined action of trypsin and carboxypeptidase B and isolated according to the method of Oyer et al. (1971). GdnHCl was ultra-pure from ICN Biochemicals. HEPES buffer was of enzyme grade from Fischer, and ethyl alcohol was anhydrous from Quantum Chemicals (200 proof). All other chemicals were of analytical grade.

Methods. Protein concentrations were determined by UV absorbance using extinction coefficients of 1.05 for insulin and 0.65 for proinsulin at 278 nm for a 1 mg/mL solution in a 1-cm path length (Frank et al., 1972b). The concentration of C-peptide was determined by quantitative amino acid analysis following acid hydrolysis. The molecular weights of insulin and proinsulin are 5808 and 9390, respectively. Denaturation samples were prepared by combining different ratios of protein and denaturant stock solutions and a solution of 20 mM HEPES, pH 7.5, 20% (v/v) ethanol, and 1 mM EDTA to obtain the desired denaturant concentration. Protein stock solutions were ~10 mg/mL insulin, 20 mM HEPES, pH 7.5, 20% (v/v) ethanol, and 1 mM EDTA with or without GdnHCl. The stock of denaturant was ~7 M GdnHCl, 20 mM HEPES, pH 7.5, 20% (v/v) ethanol, and 1 mM EDTA. The concentrations of GdnHCl were determined by refractometry (Nozaki, 1972) using an Abbe-3L refractometer. When mixing the denaturation samples, the protein stock was added last and mixed by gentle swirling of the sample solution. Since GdnHCl has a maximum solubility of ~7 M in aqueous solutions of 20% ethanol, the denaturation samples that contained >7 M were made from a stock GdnHCl solution of ~8 M that did not contain ethanol. Above 7 M GdnHCl, the circular dichroism of insulin is the same with and without ethanol. Circular dichroism measurements were made with an Aviv 62D spectrophotometer at 23 °C. All of the CD results are reported as molar ellipticity having the units degrees per molar per centimeter. The denaturation transitions were analyzed as described by Pace et al. (1986). The pre- and posttransition base lines were determined by fitting the maximum number of data points possible to give the best fit to a straight line of any slope.

Results

Association and Effect of Ethanol. The GdnHCl-induced denaturation of insulin and proinsulin was initially studied in aqueous buffers (without ethanol) at neutral pH. When near-UV CD detection was utilized, the transitions did not appear cooperative, and the pretransition base lines were impossible to determine. This was due to the detection signal being sensitive to both association and folding. The association was disrupted at lower denaturant concentrations than unfolding, but substantial overlap exists between the two phenomena. With far-UV CD detection, the denaturation transitions obtained in aqueous buffer were only slightly affected by association. This was determined by varying the protein concentration over a 30-fold concentration range. The varying protein concentration mostly affected the slope of the pretransition base line, making its determination problematic.

In order to circumvent these difficulties, a mixed aqueous/ethanol solvent was used. Figure 1A illustrates that 20% (v/v) ethanol is effective in disrupting association as determined by near-UV CD while the presence of this solvent has little effect on the secondary structure as determined by far-UV CD (Figure 1B). The slight change in the far-UV signal as ethanol was added is similar to that observed in aqueous solutions upon zinc diminution (Goldman & Carpenter, 1974). Figure 1C illustrates the effect of varying protein concentration in the absence and presence of 20% ethanol on the molar ellipticity at 275 nm. An increasing negative signal at 275 nm corresponds to increased association; 20% ethanol is effective in partially disrupting association at all concentrations of insulin investigated. Greater than 20% ethanol induced substantial changes in the far-UV CD spectrum, and less than 20% ethanol was less effective in causing dissociation (data not shown). Figure 1 suggests that 20% ethanol in aqueous solvent may provide conditions that are sufficiently dissociating, such that denaturation may be studied independently of protein concentration effects.

Denaturation of Insulin. GdnHCl-induced denaturation of insuln as detected by CD at 224 nm is shown in Figure 2A. The denaturation transition insulin symmetrical, and sigmoidal with a pretransition base line from 0 to 2 M GdnHCl and a posttransition base line from 6.5 to 8 M. The transition illustrated in Figure 2A is completely reversible (independent of starting with the protein in the native or denatured state) and independent of protein concentration from 0.05 to 1.5 mg/mL. The denaturation results reach equilibrium in less than 30 s and are stable for at least 24 h. Stability for longer periods of time was not explored. The symmetrical shape of the denaturation transition in Figure 2A is consistent with a two-state denaturation mechanism. By use of a two-state denaturation mechanism, the results of Figure 2A were quantitated by plotting as shown in Figure 2D. The abscissa of Figure 2D, K_{app}, represents the apparent equilibrium constant for the two-state unfolding reaction, native $\leftrightarrow$ denatured (K_{app} = [denatured]/[native]). K_{app} was determined by extrapolating the pre- and posttransition base lines into the

[1] Abbreviations: connecting peptide, residues 31–65 of proinsulin that connect the B chain to the A chain; C-peptide is composed of residues 33–63 and is derived from the connecting peptide of proinsulin by the combined action of trypsin and carboxypeptidase B; CD, circular dichroism; UV, ultraviolet; GdnHCl, guanidine hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

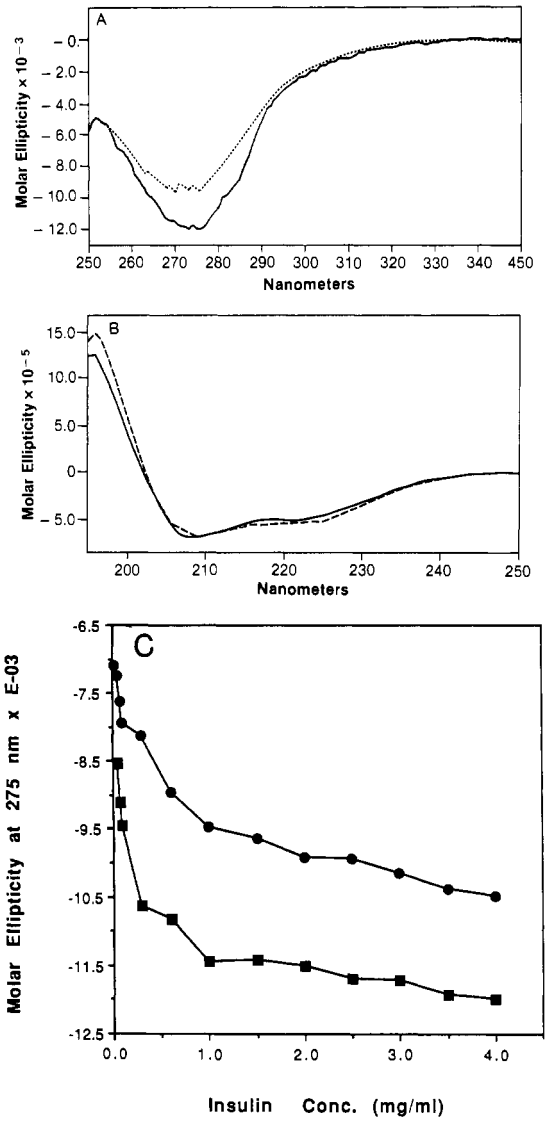

FIGURE 1: Effect of ethanol on the CD spectrum of insulin. Panel A is the near-UV and panel B is the far-UV CD spectrum of insulin in the absence (—) and in the presence of 20% ethanol (---). The protein concentration was 1.0 mg/mL. (C) Effect of varying protein concentration in the absence (■) and presence of 20% ethanol (●) on the CD at 275 nm. The CD data for panels A–C have the units degrees per molar per centimeter.

transition region, and the change from the pretransition region to the transition represents the fraction unfolded. By use of the Gibbs equation relating free energy to the equilibrium constant, the free energy of unfolding for insulin in the absence of GdnHCl was determined by extrapolating the straight line of Figure 2D to zero concentration of GdnHCl. The line for insulin in Figure 2D was determined by a least-squares analysis of the data and is defined by a slope of 1.6 and a midpoint of 5.0 which yields a free energy of unfolding of 4.5 kcal/mol. Repeated denaturation of insulin samples prepared separately established a variation of ±0.5 kcal/mol for the free energy of unfolding and ±0.1 M GdnHCl for the midpoint of denaturation.

Denaturation of insulin was also detected by circular dichroism at 275 nm (Figure 2B). As can be seen in Figure 2D, the denaturation curves detected at 224 and 275 nm are coincident. This indicates that denaturation of insulin is a cooperative process with the secondary and tertiary structures denaturing simultaneously. The coincidence of the denaturation transitions, as detected by CD at 224 and 275 nm, is consistent with a two-state mechanism for insulin denaturation (i.e., if stable intermediates were populated with nonnative optical properties, then noncoincident transitions for the different wavelengths of detection might be expected).

Denaturation of Proinsulin. Shown in Figure 2A is the equilibrium denaturation of proinsulin as detected by far-UV CD. In comparison to insulin, the denaturation transition for proinsulin is multiphasic and not consistent with a two-state mechanism of unfolding. Denaturation of insulin and proinsulin differ in the 0–2.5 M GdnHCl concentration range. Since the chemical difference between insulin and proinsulin is the connecting peptide, we investigated the conformation of isolated C-peptide. In the absence of denaturant, isolated C-peptide has a small degree of ordered secondary structure according to the far-UV CD (Frank et al., 1972a). Shown in Figure 2C is the GdnHCl-induced denaturation of isolated C-peptide. Addition of GdnHCl causes the C-peptide structure to unfold in a cooperative and reversible manner. The denaturation results of isolated C-peptide were subtracted from the results obtained for proinsulin to obtain the data for corrected proinsulin shown in Figure 2A,D. After the proinsulin transition was adjusted for the connecting peptide contribution, a similar denaturation transition to insulin is obtained (Figure 2A,D). These results show that connecting peptide, either isolated or as part of proinsulin, is not random coil and represents an autonomous folding domain; furthermore, the insulin domain of proinsulin is indistinguishable from insulin according to conformational stability.

The denaturation of proinsulin as detected at 275 nm is illustrated in Figure 2B. The denaturation results for insulin and proinsulin are identical at 275-nm detection.

Discussion

The equilibrium denaturation results presented here (Figure 2A,B) demonstrate that all the information required for insulin to fold is contained within its amino acid sequence. Although this has been known for some time by inference from the A- and B-chain combination work (Chance et al., 1981), it is often misstated in the literature that only the insulin precursor, proinsulin, will refold. It is clear that the connecting peptide portion is not absolutely required for folding.

The presence of folding intermediates is manifested in equilibrium denaturation measurements by nonsymmetrical transitions or by the noncoincidence of transitions when detected by different probes (Kim & Baldwin, 1982). Denaturation of insulin does not manifest either of these criteria for intermediates. However, proinsulin denaturation detected at 224 nm displays a nonsymmetrical transition. This is due to the connecting peptide portion of proinsulin having secondary structure that is independent of the insulin portion. A proinsulin equilibrium intermediate represents a partly unfolded species with the connecting peptide portion unfolded and the insulin portion folded. The denaturation transition of proinsulin detected at 275 nm is identical with that of insulin. Since there are no aromatic amino acids in the connecting peptide (Steiner et al., 1971), its unfolding is optically silent at 275 nm. The results of Figure 2C show that isolated C-peptide has ordered structure as determined by far-UV CD, even in the absence of insulin. The coincidence of the denaturation curves for insulin and proinsulin [after adjusting for the connecting peptide contribution (Figure 2A,D) or if detected at 275 nm (Figure 2B)] shows that the insulin portion of proinsulin has the same insulin-like conformational stability. This finding is consistent with previous observations that the CD spectrum of an equimolar mixture of insulin and C-peptide was within experimental error equal to the CD spectrum of proinsulin (Frank et al., 1972a). The decreased activity of

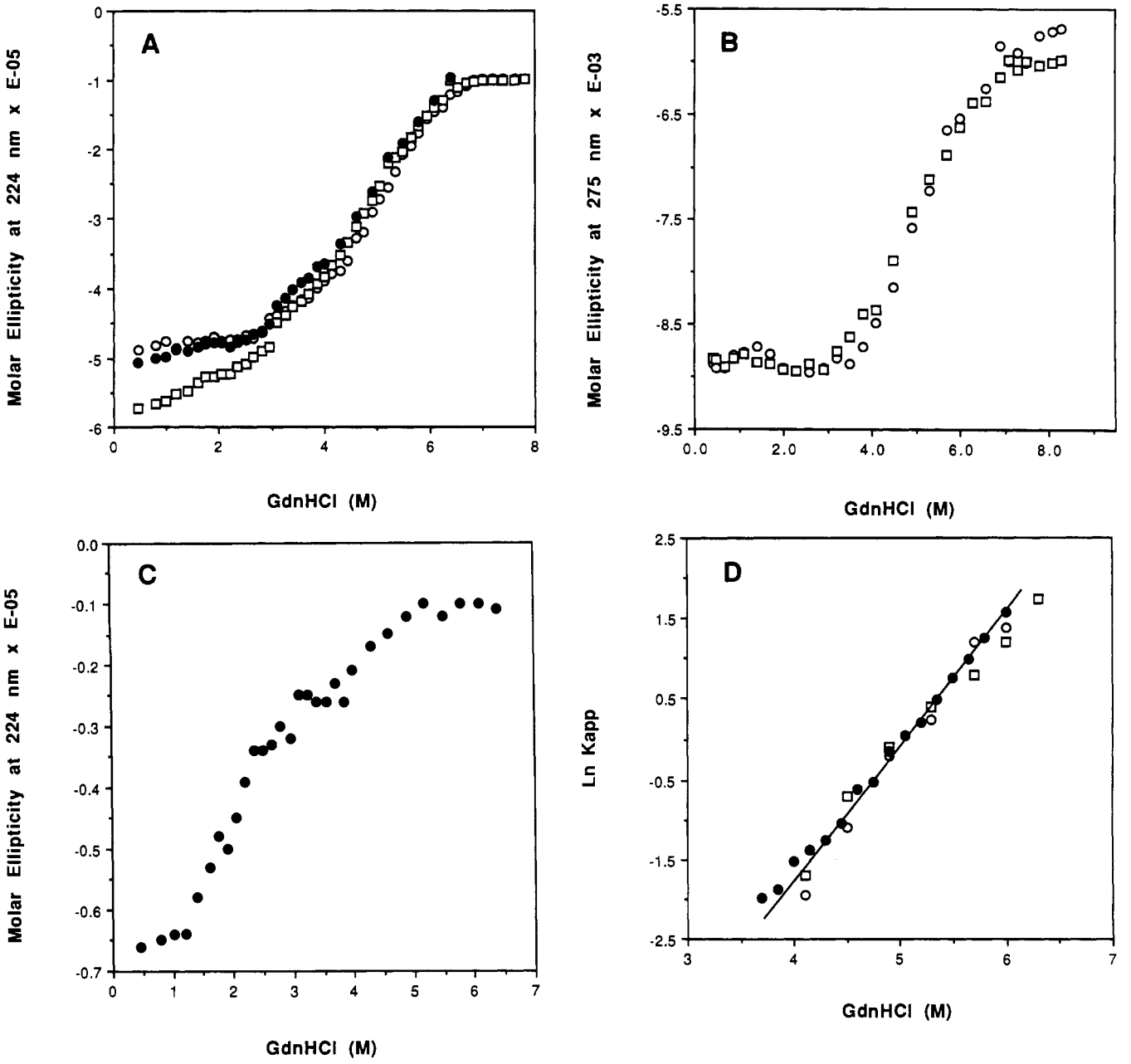

FIGURE 2: GdnHCl-induced equilibrium denaturation. (A) Detection by far-UV CD: (○) results for insulin, (□) proinsulin, and (●) proinsulin after subtracting the contribution of connecting peptide as determined from Figure 2C. The insulin concentration was 0.54 mg/mL (92.5 μM), [proinsulin] was 0.93 mg/mL (99 μM), and [C-peptide] was 0.69 mg/mL (193 μM). (B) Detection by near-UV CD: (○) results for insulin and (□) proinsulin. The insulin concentration was 1.05 mg/mL (0.17 mM), and [proinsulin] was 1.70 mg/mL (0.18 mM). (C) Far-UV CD-detected denaturation of C-peptide. The C-peptide concentration was 0.69 mg/mL (193 μM). (D) The natural logarithm of the apparent equilibrium constant for unfolding. The apparent equilibrium constant was calculated from the data in panels A and B by using K_{eq} = fraction unfolded/(1 − fraction unfolded). The straight line was determined by a least-squares fit to the data. (○) Insulin at 275 nm; (●) insulin at 223 nm; (□) proinsulin at 275 nm.

proinsulin (10–20%) is probably due to the lack of the free N-terminus of the A chain and not to an altered conformation of the insulin region. Numerous studies, including chemical modification, have corroborated the importance of the A-chain α-amino group (Pullen et al., 1976; Rosen et al., 1980) for receptor binding. The exact type of ordered structure observed in the C-peptide is not clear at this point. At room temperature, the molar ellipticity at 220 nm is ~65000 deg M^{-1} cm^{-1}, which is relatively weak. This might suggest a marginally stable structure that is only partly folded or that only a small section of the peptide is ordered. For example, a β-sheet of six residues, with the remaining being random coil, would account for the CD signal observed. It is interesting to speculate that the connecting peptide conformation may play some functional role in the storage or processing of proinsulin. The lower conformational stability of the connecting peptide portion of proinsulin could make this region more accessible to proteolysis which would aid in the conversion of proinsulin into insulin.

The denaturation results reported in Figure 2A,B are highly reproducible. Repetitive measurements of separate sample preparations show a variation of only ±0.5 kcal/mol and ±0.1 M GdnHCl for the free energy of unfolding and the midpoint of the denaturation transition, respectively. The inclusion of alcohol was effective in eliminating the complications due to protein aggregation. Ethanol was utilized because of its minimal effect on the monomeric structure of insulin, and insulin is capable of binding to receptor in 20% ethanol

(personal communication from Michael Weiss and Steven Shoelson, Harvard Medical School). Figure 1 demonstrates that the far-UV CD spectrum is minimally affected by 20% ethanol, whereas the near-UV CD is altered by ethanol because the near-UV CD spectrum is sensitive to association. The decrease in the negative band at 275 nm that results from 20% alcohol (Figure 1A) is due to dissociation of insulin. The dissociating effect of ethanol is consistent with the X-ray crystallography data which show that hydrophobic bonding is the main source of interaction between the multimers (Baker et al., 1988).

The effect of Zn and the resulting hexamer formation on the equilibrium denaturation of insulin was explored (data not shown). This was done in the absence of ethanol and with an equimolar concentration of Zn to insulin. With denaturation detected at 224 nm, there was no significant effect of Zn. The denaturation results obtained by detection at 275 nm were impossible to compare to the absence of Zn because of the overlap between dissociation of hexamer and unfolding which both result in signal changes at this wavelength.

The mixed organic/aqueous solvent utilized in this study makes it inappropriate to rigorously relate the denaturation results obtained for insulin to other proteins. However, as shown here, this method is valid for comparing two related molecules like insulin and proinsulin or for comparing the same molecule by multiple detection methods. We intend to utilize this denaturation system in the future to assess the contribution of certain amino acids to the conformational stability of insulin.

Registry No. Insulin, 9004-10-8; proinsulin, 9035-68-1; proinsulin C-peptide, 59112-80-0; ethanol, 64-17-5.

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