Thermal Dissociation and Unfolding of Insulin

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ABSTRACT: The thermal stability of human insulin was studied by differential scanning microcalorimetry and near-UV circular dichroism as a function of zinc/protein ratio, to elucidate the dissociation and unfolding processes of insulin in different association states. Zinc-free insulin, which is primarily dimeric at room temperature, unfolded at ∼70 °C. The two monomeric insulin mutants Asp^{B28} and Asp^{B9},Glu^{B27} unfolded at higher temperatures, but with enthalpies of unfolding that were approximately 30% smaller. Small amounts of zinc caused a biphasic thermal denaturation pattern of insulin. The biphasic denaturation is caused by a redistribution of zinc ions during the heating process and results in two distinct transitions with T_m's of ∼70 and ∼87 °C corresponding to monomer/dimer and hexamer, respectively. At high zinc concentrations (≥5 Zn^{2+} ions/hexamer), only the hexamer transition is observed. The results of this study show that the thermal stability of insulin is closely linked to the association state and that the zinc hexamer remains stable at much higher temperatures than the monomer. This is in contrast to studies with chemical denaturants where it has been shown that monomer unfolding takes place at much higher denaturant concentrations than the dissociation of higher oligomers [Ahmad, A., et al. (2004) *J. Biol. Chem*. *279*, 14999−15013].

Insulin is a protein hormone consisting of an A-chain (21 residues) and a B-chain (30 residues) linked by two disulfide bonds. In addition, the A-chain contains an intrachain disulfide bond. In the bloodstream, insulin is present at very low concentrations. At these low concentrations, insulin is monomeric, which is its biologically active form. At higher concentrations, insulin assembles into dimers and, in the presence of zinc ions at neutral pH, into hexamers (*1*). Each of two zinc ions binds to three His^{B10} residues, and this arrangement is pivotal for insulin hexamer formation. In insulin crystals, up to 10 additional zinc ions bind in other sites. It is not clear if these additional sites are also found in insulin hexamers in solution (*2*−*5*). The hexamer is known to exist in three different conformations which have been given the nomenclature T_6, T_3R_3, and R_6 depending on the conformations of the monomer subunits. The principal difference is that residues B1−B8 are converted from an extended conformation in the T-state to an α-helix in the R-state. In addition to zinc, ligands binding to allosteric sites on the hexamer are required to change conformation from the T- to the R-state (*6*).

The structure and stability of insulin have been the subject of numerous studies since the introduction of insulin as a drug in the 1920s. The physical and chemical stability of insulin is, among many other parameters, strongly influenced by the association state and the conformation of the protein, the hexamer being the most stable form (*7*).

It has been known since 1922 that the biological activity of insulin is destroyed upon boiling (*8*, *9*). Since then, several studies have involved the effects of heat on insulin. Most of these studies have addressed the fibrillation of insulin and have been performed in acidic solvents under conditions where insulin is mainly in equilibrium between monomers and dimers. CD[1] and FT-IR data of bovine insulin at pH 2.6 heated to 68 °C for 2 h indicated a partially unfolded structure with a reduced content of α-helix and the appearance of extensive β-sheet structure (*10*). The results are confirmed by a recent study which supplemented CD studies with ^1H NMR and $^1H-^{15}N$ NMR studies. The authors propose a novel partial fold (at 60 °C and pH 2.4) in which the N-terminal segments of the A- and B-chains detach from the core and the N-terminal α-helix of the A-chain is unfolded (*11*). Bovine insulin at pH 3 (1 mg/mL) has been examined by FUV and NUV CD at temperatures from 27 to 70 °C. At 27 °C, the insulin existed in a state with a monomer−dimer equilibrium. The magnitude of the characteristic NUV CD band at 276 nm that is related to the monomer−monomer interaction in the dimer was decreased upon heating. At 70 °C, less than 50% of the signal remained, indicating a dissociation of the dimer into monomers (*12*). Heat-induced β-elimination of cystine residues has been assessed in bovine insulin. The half-life (first-order kinetics) of disulfide destruction at 100 °C at pH 8 and 6 was 0.67 and 9.3 h, respectively (*13*).

The aggregation of bovine insulin at pH 1.9 (20 mg/mL in HCl) has been studied by differential scanning calorimetry

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[1] Abbreviations: DSC, differential scanning calorimetry; CD, circular dichroism; FUV, far-UV; NUV, near-UV; T_m, transition midpoint; GdnHCl, guanidine hydrochloride.

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(DSC) (*14*). Under these conditions, bovine insulin is primarily dimeric (*15*). The DSC scans showed one reversible endothermic transition with a T_m at 60 °C immediately followed by an exothermic peak. FTIR spectroscopy suggested a molten globule-like intermediate occurring at the T_m and preceding further unfolding to an aggregation-competent unstructured intermediate (*14*). Recently, the exothermic nature of insulin aggregation has been confirmed, and it has been shown that the ΔC_p of the aggregation is negative. This is in contrast to unfolding which typically gives a positive ΔC_p (*16*).

To the best of our knowledge, there are no studies examining the events that take place during thermal denaturation of hexameric human insulin near neutral pH. This is of special interest, since insulin as a drug is most commonly formulated under hexamer stabilizing conditions at near-neutral pH. From the information given above, it is expected that heating of hexameric human insulin will lead to a complex combination of several events, including dissociation, unfolding (possibly with intermediate states), non-native aggregation, and disulfide destruction and reshuffling. In addition to disulfide destruction, there are many other temperature-dependent routes of chemical degradation, notably, hydrolysis and formation of high-molecular weight products (*17*−*19*). The aim of this work is to study dissociation and unfolding during thermal denaturation of hexameric human insulin. DSC and CD studies are applied to differentiate between dissociation and unfolding events.

MATERIALS AND METHODS

Materials. Zinc-free native human insulin, zinc-free insulin Asp^{B28}, and zinc-free insulin Asp^{B9},Glu^{B27} were obtained from Novo Nordisk A/S. All other chemicals were analytical grade. Deionized water was filtered using a Millipore system (Millipore, Billerica, MA) and used for all samples. All insulin samples were prepared in 7 mM phosphate buffer adjusted to pH 7.4 with perchloric acid and/or sodium hydroxide. All insulin samples were made from fresh stock solutions to give a concentration of 0.6 mM. The final concentration was determined from the absorbance at 276 nm using an ϵ_{276} of 6200 M^{-1} cm^{-1} (*20*).

Differential Scanning Calorimetry (DSC). Data collection was performed using a VP-DSC differential scanning microcalorimeter (MicroCal, LLC, Northampton, MA) (*21*). All protein scans were performed with 7 mM phosphate buffer in the reference cell from 25 to 110 °C at a scan rate of 1 °C/min (unless otherwise stated) and an excess pressure of 0.21 MPa. All samples and references were degassed immediately before use. A buffer−buffer reference scan was subtracted from each sample scan prior to concentration normalization. Data analysis was carried out using Origin 7.0 (OriginLab, Northampton, MA). For independent non-two-state transitions, curve fitting was carried out using eqs 1 and 2:

$$C_p(T) = \frac{K(T)\Delta H_{vH}\Delta H}{[1 + K(T)]^2 RT^2} \quad (1)$$

$$K(T) = \exp\left[\frac{-\Delta H_{vH}}{RT}\left(1 - \frac{T}{T_m}\right)\right] \quad (2)$$

where C_p is the heat capacity, T the temperature, K the equilibrium constant of unfolding, ΔH the calorimetric enthalpy, ΔH_{vH} the van't Hoff enthalpy, R the gas constant, and T_m the transition midpoint. For the data analyses of the zinc-free insulin mutants, DSCfit software was used. The thermodynamic functions applied by DSCfit are described in a paper by Grek et al. (*22*). DSCfit was used in the cases where the results were significantly dependent on the user-defined baseline creation in Origin. One advantage of DSCfit software is that the baselines are automatically generated. For comparison, both methods were applied to the zinc-free human insulin samples where the baseline creation in Origin was unmistakable.

Circular Dichroism (CD). Near-UV circular dichroism spectra were obtained on a Jasco J-715 circular dichroism spectrophotometer (Jasco, Tokyo, Japan). The filtered insulin samples were scanned in a 0.1 cm cell from 300 to 250 nm using a bandwidth of 2.0 nm, a response time of 2 s, a data pitch of 0.5 nm, and a scanning speed of 20 nm/min. A Peltier element was used to control the heating of the CD samples during temperature scans. Each spectrum is the accumulation of two scans. Spectra of the buffer were recorded and subtracted from each sample spectrum. The protein concentration obtained by UV absorbance was used to calculate the molar ellipticity (θ) on a per residue basis.

RESULTS

DSC with Native Human Insulin. The behavior of insulin in thermal denaturation by DSC was investigated. The effects of varying the scan rate for zinc-free insulin and zinc-bound insulin (5.2 Zn^{2+} ions/hexamer) are shown in Figure 1. The thermograms depict the excess heat capacity of the sample relative to a reference (buffer). Scan rates of 30, 60, and 90 °C/h give overlapping thermograms of zinc-free insulin when looking at the main endotherm, and the transition midpoints (T_m) are the same. However, an exotherm, which is not observed at a scan rate of 90 °C/h, gradually appears at 100−110 °C when using slower scan rates. At the slowest scan rate (10 °C/min), this exotherm appears even before the endotherm has been completed, and the completion of the exotherm is reached just above 100 °C, where the excess heat capacity returns to zero. The scan rate variation with zinc−insulin samples (see Figure 1B) shows a decrease in T_m with a decrease in scan rate. The decrease is quite small but nevertheless indicative of a kinetically controlled denaturation. For the following DSC studies, a scan rate of 60 °C/h was chosen to stay clear of the exothermal event and to optimize the sensitivity to slow equilibria and kinetic processes.

The reversibility of the transitions was investigated by performing two consecutive DSC scans on the same sample. Figure 1C shows the reheating of zinc-free and zinc-containing samples after the performance of an initial scan to near completion of the transition. For both samples, the area under the peak of the second scan is reduced by approximately 20% compared to the first scan. Thus, both transitions are only partly reversible.

Initial DSC studies were performed with human insulin containing 2−3 zinc ions per hexamer which is an amount often used when studying hexameric insulin. Under these conditions, it was found that the thermal denaturation by DSC

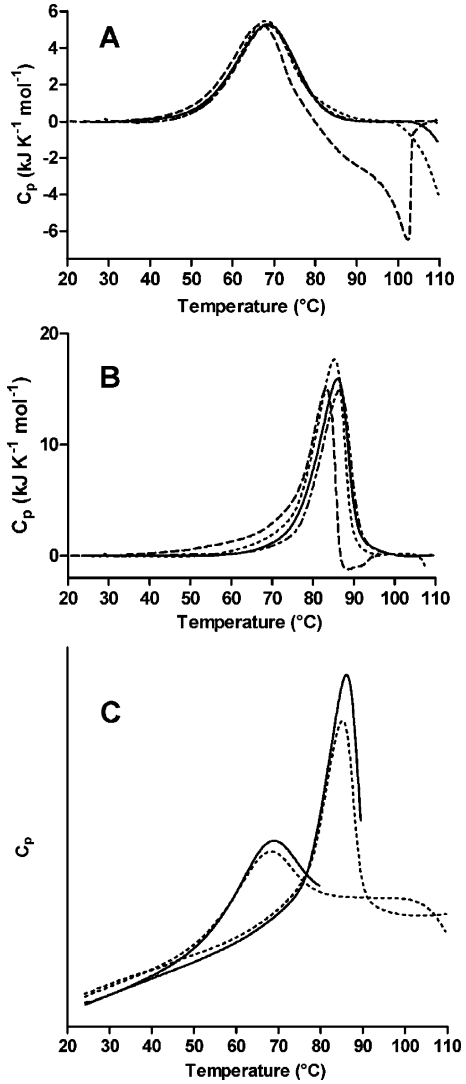


FIGURE 1: DSC scans with varying scan rates of human insulin in 7 mM phosphate buffer at pH 7.4: zinc-free (A) and with 0.52 mM Zn^{2+} (B). The dashed line represents a rate of 10 °C/h, the dotted line a rate of 30 °C/h, the solid line a rate of 60 °C/h, and the dashed and dotted line a rate of 90 °C/h [coincides with the solid line (60 °C/h) and is therefore hardly visible]. (C) DSC thermograms of repetitive scans of zinc-free insulin (low T_m) and zinc-bound insulin (0.5 mM Zn^{2+}, high T_m). Both samples are 0.6 mM human insulin in 7 mM phosphate buffer at pH 7.4: first scan, full line; second scan, dotted line.

exhibited two apparent transitions. The DSC thermograms showed two distinct peaks at 72 and 86 °C. DSC scans with varying amounts of zinc were performed to investigate the effect of zinc on the two apparent thermal transitions (see Figure 2). Zinc-free 0.6 mM human insulin at pH 7.4 exhibits one apparent transition with a transition midpoint (T_m1) at 68.5 °C. In the presence of 0.1 mM Zn^{2+}, a second transition appears at around 84 °C (T_m2). When the amount of Zn^{2+} in further increased, the transition with T_m1 gradually disappears while the transition with T_m2 increases. At ≥0.5 mM Zn^{2+}, only the transition with T_m2 (87.6 °C) is present. The results show a clear change in the thermal denaturation when going from the primarily dimeric state of the zinc-free insulin to the stable hexamers formed upon addition of excess amounts of Zn^{2+}.

The DSC results could be fitted to different models according to the association state. The thermogram for zinc-free insulin fitted poorly to a simple two-state transition, but the fit to a non-two-state model was excellent (see Figure 3). Insulin with saturating amounts of zinc did not converge with any of the simple models but exhibited an excellent fit to a two-state model with subunit dissociation. The calculated amount of subunits (n) in the fitting procedure depended strongly on the choice of baseline setting. Therefore, this parameter is not reliable and not reported here.

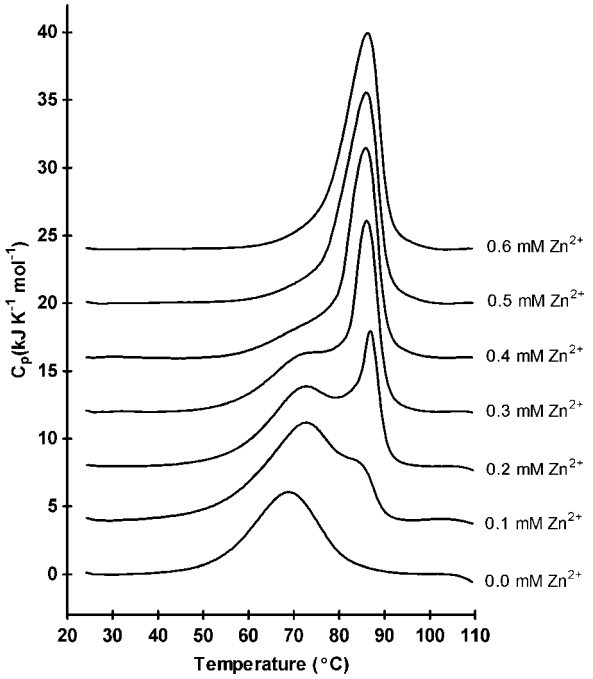


FIGURE 2: DSC thermograms of 0.6 mM human insulin in 7 mM phosphate buffer at pH 7.4. The Zn^{2+} concentration of the samples is indicated next to each thermogram. Buffer−buffer scans have been subtracted from each scan which has then been concentration normalized and baseline adjusted.

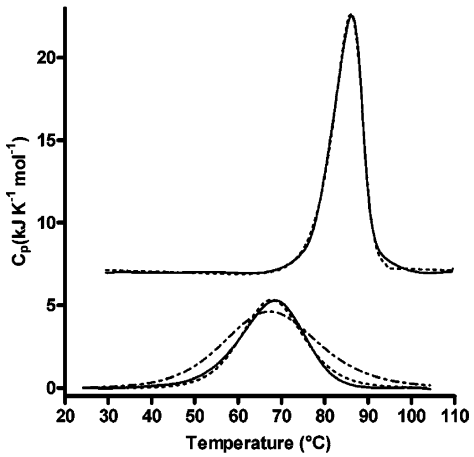


FIGURE 3: Fitting of the experimental DSC data (solid lines) to models using Origin 7.0 (OriginLab). The top graph shows the fitting of insulin with 5 Zn^{2+} ions/hexamer to a two-state model with subunit dissociation (dotted line). The bottom graph shows the fitting of zinc-free insulin to a two-state transition (dashed and dotted line) and to a non-two-state transition (dotted line).

DSC with Insulin Analogues. DSC studies were conducted with the two monomeric insulin mutants Asp^{B28} and Asp^{B9},Glu^{B27}. The mutants were studied in an effort to compare the thermal denaturation of the insulin monomer with the insulin dimer. Zinc-free native insulin is primarily dimeric under the conditions used in this study. At 1 mM

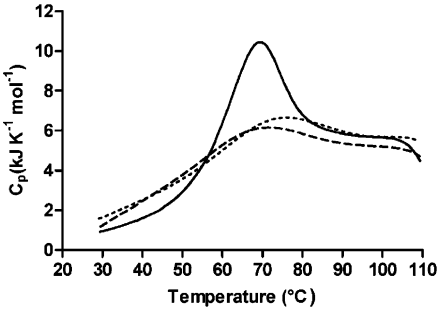


FIGURE 4: DSC thermograms of 0.6 mM zinc-free insulin in 7 mM phosphate buffer at pH 7.4: solid line, human insulin; dashed line, insulin Asp^{B28}; and dotted line, insulin Asp^{B9},Glu^{B27}.

(and zinc-free in 20 mM Tris and 100 mM NaCl), it has been found that the association state of Asp^{B28} and Asp^{B9},Glu^{B27} is 1.4 and 1.1, respectively, where 1.0 is 100% monomer and 6.0 is 100% hexamer. Under the same conditions, the association state of native human insulin was found to be 4.4. Thus, the insulin dimers associate to some degree but do not form stable hexamers (*20*). In another study, the association states of the same mutants at a concentration of 0.34 mM in 20 mM phosphate and 100 mM NaCl at pH 7.4 were studied. The association state was 1.25 for Asp^{B28} and 1.30 for Asp^{B9},Glu^{B27} (*15*). DSC thermograms of the mutants are compared with zinc-free human insulin in Figure 4. Interestingly, the T_m's of the monomeric mutants are higher than the T_m of the dimeric native insulin, while the enthalpy of unfolding (ΔH_u) of the dimer is greater than the ΔH_u of the two monomeric mutants. Results of thermodynamic calculations for the two insulin mutants, zinc-free native insulin, and hexameric zinc-bound insulin (5 Zn^{2+} ions/hexamer) are compiled in Table 1. The stabilizing effect of zinc on native insulin causes a 20 °C increase in T_m which is complemented by a 50% increase in ΔH_u compared to that of zinc-free native insulin. The ratios between the van't Hoff enthalpy (ΔH_{vH}) and ΔH_u are indications of the association states of zinc-free native insulin and the monomeric mutants. Data analysis of the zinc free samples showed a negative ΔC_p at the T_m and nonrandom residual scatter of the fit (data not shown) which is indicative of non-two-state processes (*22*).

NUV CD. The DSC transitions represent the thermal effects of both unfolding and dissociation of hexamers and dimers. NUV CD thermal scans were performed to study the dissociation of human insulin with 0−6 Zn^{2+} ions/hexamer. Insulin dimers and hexamers exhibit a characteristic tyrosyl CD signal at ∼276 nm. Main contributors to this signal are interactions in the monomer−monomer interface of the dimers and in the dimer−dimer interface of the hexamers (*23*, *24*).

The effect of increasing temperature on the NUV CD spectra (300−250 nm) is a marked decrease in the tyrosyl CD signal. The spectrum at 20 °C (see Figure 5A) is characteristic of insulin hexamers. With an increase in temperature, the magnitude of the 276 nm peak gradually diminishes, and at 90 °C, it has completely disappeared.

NUV CD thermal scans of 0.6 mM native human insulin with increasing amounts of Zn^{2+} and the two monomeric mutants are shown in Figure 5B. At the starting point (20 °C), the negative CD signal at 276 nm increases markedly when Zn^{2+} is added as a manifestation of the increasing

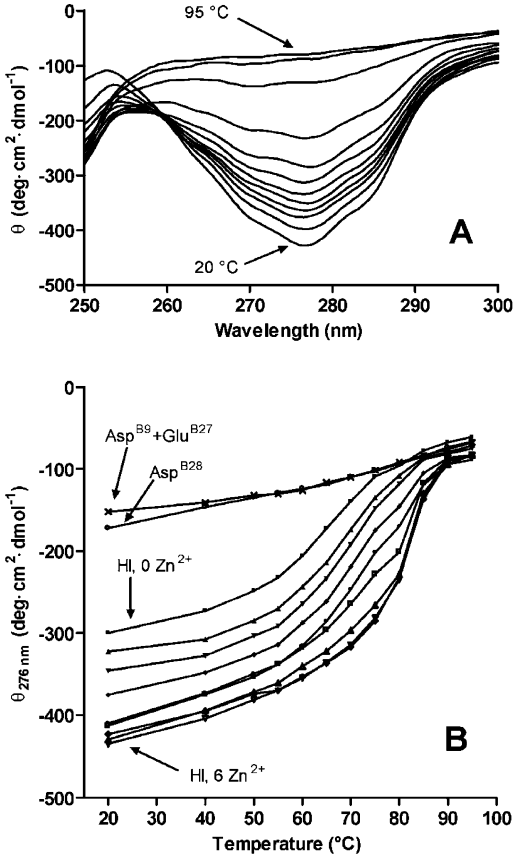


FIGURE 5: (A) NUV CD spectra of 0.6 mM insulin with 0.5 mM Zn^{2+} at varying temperatures. From the bottom (largest negative signal) to the top, temperatures range from 20 to 95 °C. (B) Thermal scanning of ellipticity in the NUV range. The ellipticity at 276 nm is shown as a function of temperature. Thermal scans were performed with 0.6 mM human insulin in 7 mM phosphate buffer at pH 7.4 with varying amounts of Zn^{2+}. From the top to the bottom curve, the Zn^{2+} concentration is 0, 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, and 0.6 mM. Thermal scans with zinc-free insulin AspB^{28} and Asp^{B9},Glu^{B27} in 7 mM phosphate buffer at pH 7.4 are indicated in the graph.

hexamer content. The zinc-free scan shows a transition beginning around 50 °C with a midpoint of approximately 65 °C. With increasing amounts of zinc, it can be seen that the transition appears at higher temperatures. The scans with 0.5 and 0.6 mM Zn^{2+} coincide, and the transition midpoint has increased to 80−85 °C. The transition represents dissociation of hexamers and dimers to monomers. Thus, the zinc-free scan represents dissociation of dimers, and the scans with 0.2−0.6 mM Zn^{2+} represent dissociation of hexamers. Samples with 0.05−0.2 mM Zn^{2+} contain substoichiometrical amounts of Zn^{2+}, and the NUV CD transition therefore represents a combination of hexamer and dimer dissociation. Samples with 0.15−0.4 mM Zn^{2+} show a biphasic transition. The transition curves exhibit a slight but reproducible decrease in the slope at 75−80 °C, immediately followed by a sharp increase in the slope from 80 to 95 °C. This decrease in slope matches the temperature interval between the two peaks in DSC thermograms (Figure 2).

DISCUSSION

The purpose of this work was to investigate the thermal denaturation of insulin in different association states. The thermal denaturation is a complex, partly reversible process

Table 1: Transition Midpoints and Enthalpies of Unfolding and Dissociation of 0.6 mM Insulin in 7 mM Phosphate Buffer at pH 7.4[a]

insulin	T_m (°C)	ΔH_u (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{vH}/\Delta H_u$
Asp^{B9},Glu^{B27}, zinc-free[b]	77.4	69.8	100.5	1.4
Asp^{B28}, zinc-free[b]	73.3	67.4	80.2	1.2
native, zinc-free ($n = 3$)	68.7 ± 0.3[b]	94.8 ± 2.5[b]	206.7 ± 9.8[b]	2.2 ± 0.15[b]
	67.7 ± 0.4[c]	100.6 ± 0.3[c]	207.2 ± 2.9[c]	2.1 ± 0.03[c]
native, 5 Zn^{2+} ions/hexamer	88.0[c]	144.4[c]	nd[d]	

[a] Enthalpy results are on a per monomer basis. [b] Data analyses performed with DSCfit software. [c] Data analyses performed with Origin software. [d] The unfolding−dissociation model used for the zinc−insulin system does not include ΔH_{vH}.

which is highly dependent on the association state of insulin. Initial DSC studies of zinc-bound insulin (2 Zn^{2+} ions/hexamer) at neutral pH revealed a complex thermogram with at least two different endothermic transitions. The aim of this work was to elucidate the molecular origin of the different peaks observed in insulin DSC studies at pH 7.4.

Zinc-Free Insulin. DSC experiments with zinc-free insulin gave thermograms with only one endotherm transition. At the low scan rate (10 °C/h), the endotherm was directly followed by an exotherm. This thermal denaturation profile is comparable to that obtained with dimeric insulin at pH 1.9−2.0 (*14*, *16*). The endothermic transition of zinc-free human insulin at pH 7.4 was shown to be a non-two-state process. This is in accordance with studies of insulin denaturation at low pH. The results indicated that the endothermic unfolding proceeds via an intermediate molten globule-like conformational state, followed by unfolding of the native secondary structure (*14*). The subsequent exothermic transition is an irreversible aggregation of the protein (*14*, *16*).

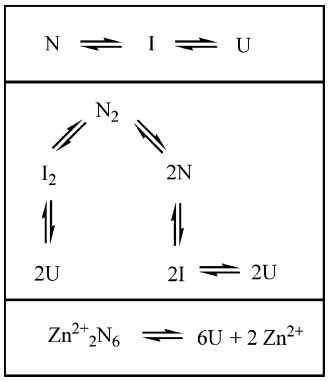
Two monomeric insulin mutants (Asp^{B28} and Asp^{B9},Glu^{B27}) were studied in an effort to compare the thermal denaturation of dimers with monomers. Thermograms from DSC scans of the mutants are compared with that of native insulin in Figure 4. In all cases, one endothermic peak is observed, and as for native insulin, the denaturation appears to follow a non-two-state process. The non-two-state unfolding of the monomer and dimer is supported by several studies showing an intermediate state of the monomer as well as the dimer (*11*, *14*, *25*−*28*). The monomeric mutants exhibit higher T_m values than native human insulin, while the ΔH of unfolding for native insulin is higher than for the two mutants. The NUV CD thermal scan of zinc-free native insulin showed that dimer dissociation takes place in the same temperature interval (50−80 °C) as the endothermic transition of the DSC scan. Thus, the thermal unfolding of insulin monomers and dimers is qualitatively comparable, but it appears that dissociation of the dimer upon heating increases the ΔH of denaturation. Unfolding studies with guanidine hydrochloride (GdnHCl) have shown that the Asp^{B9} mutation has no effect on the stability of the monomer fold (*28*). GdnHCl unfolding studies of Asp^{B28} and Asp^{B9},Glu^{B27} have shown stability similar to that of native human insulin (unpublished results, oral communication with N. Kaarsholm). Therefore, it is reasonable to assume that the ΔH_u of monomeric native insulin is similar to those of the monomeric mutants.

Zinc Binding and Biphasic Denaturation. Addition of zinc to insulin induces and stabilizes the hexamer. At 25 °C, at pH 7.5, and in the presence of 2 Zn^{2+} ions/hexamer, native insulin has been shown to be completely hexameric down to a concentration of 0.2 mM (*29*). However, with 2 Zn^{2+} ions/hexamer and 0.6 mM insulin at pH 7.4, the DSC thermogram indicates unfolding of more than just one species. The first apparent transition has a T_m (∼70 °C) consistent with the thermogram of the zinc-free dimeric insulin. Increasing the zinc concentration diminishes the magnitude of the first peak, and at 5 Zn^{2+} ions/hexamer, the dimer peak is not observed. The peak observed with 5 Zn^{2+} ions/hexamer has a T_m of 88.0 °C. This kind of ligand-induced multiphasic thermal protein denaturation has been demonstrated for some other proteins. A biphasic denaturation of defatted human albumin has been demonstrated upon addition of high-affinity ligands (*30*). A similar thermal denaturation process has been demonstrated for ribonuclease A (RNase A). Binding of 2′-CMP, which binds 1:1 in the active site of RNase A, changes the thermal denaturation from a simple two-state transition to a biphasic denaturation (*31*, *32*). Recently, biphasic thermal denaturation has also been demonstrated with riboflavin binding protein in the presence of the ligand flavin mononucleotide (*33*). Ligand-induced biphasic denaturation can occur when (1) the initial concentration of bound ligand is substantially greater than that of the free ligand, (2) the protein is initially subsaturated with ligand, and (3) the ligand binding affinity for the denatured protein is substantially smaller than for the native protein. During denaturation, the free ligand concentration increases due to the release of bound ligand by unfolding protein. The total ligand concentration is unchanged, but the ligand saturation level of the remaining native protein is increased. Theoretical thermodynamic descriptions of the phenomenon have been proposed by several authors (*31*, *32*, *34*, *35*).

In the simple case of a single ligand-binding site on a native monomeric protein, a low binding affinity (10^4 M^{-1}) results in only a single, symmetric endotherm. At a higher affinity (10^6 M^{-1}), a biphasic thermogram may be observed (*34*). The affinity of zinc for insulin has been found to be in the high-affinity range. At pH 8, the affinity of zinc for bovine insulin was 1.86×10^5 M^{-1} at 30 °C (*3*) and 1.9×10^6 M^{-1} at pH 7 up to 1.7 Zn^{2+} ions/hexamer and ∼100 times lower for the following binding of 1.7−6.0 Zn^{2+} ions/hexamer (*2*). Similarly, at pH 8.0, two main orders of zinc binding were found with a K_1 of 4.7×10^6 M^{-1} and a K_2 of 3.5×10^4 M^{-1}, where the first 2.4 Zn^{2+} ions are bound with K_1 and the following 2.4−10.2 Zn^{2+} ions are bound with K_2 (*4*). Thus, biphasic denaturation of the zinc−insulin system is feasible.

Whereas the above-mentioned examples of ligand-induced biphasic denaturation are all binding of a ligand to a monomeric protein, the mechanism for insulin is more complex. The binding of zinc causes formation of stable insulin hexamers. The endotherm of the hexamers involves both dissociation and unfolding, whereas the endotherm of the zinc-free insulin does not show dissociation behavior.

Scheme 1: Proposed Mechanism for Thermal Denaturation of the Insulin Monomer (top), Dimer (middle), and Zinc Hexamer (bottom)[a]



[a] N stands for native insulin, I for intermediate state(s), and U for unfolded insulin.

Thus, zinc binding induces oligomerization as well as stabilization, and the endotherms in the biphasic denaturation are qualitatively different. No theoretical thermodynamic models are available that describe systems with this complexity.

Model for Thermal Denaturation of Insulin. Hexamer unfolding is best fitted to a model incorporating dissociation, while the zinc-free insulin unfolding can be fitted to a single non-two-state transition. However, the zinc-free insulin is primarily dimeric, and therefore, one would also expect dissociation to be involved in the thermal denaturation. NUV CD studies were performed to distinguish dissociation from unfolding. The DSC studies with the monomeric mutants showed that monomer and dimer denaturation occurred in the same temperature range. The NUV CD studies of zinc-free native insulin showed that dimer dissociation occurs in exactly the same temperature range as the monomer unfolding by DSC. From these results, it is therefore not possible to conclude what happens first to the dimer, dissociation or unfolding, or whether both processes take place simultaneously. The NUV CD thermal scan of the zinc−insulin system showed that the dissociation of the hexamer also takes place in the same temperature range as denaturation observed by DSC. However, since the hexamers denaturate at temperatures above the T_m of dimer denaturation, it is evident that the dissociation of the hexamer is the limiting step for denaturation of the insulin hexamer. A model for thermal denaturation of insulin is proposed in Scheme 1. The temperature at which the hexamer dissociates is so high that unfolding of the monomer occurs instantaneously and no intermediate states are populated. Therefore, the hexamer denaturation can be described by a two-state mechanism going directly from the hexamer to the denaturated monomer.

Thermal Denaturation versus Chemical Denaturants. Numerous guanidine hydrochloride (GdnHCl) denaturation studies with native insulin and insulin mutants have been reported in the literature. Most of these studies involve the unfolding of the monomer under various conditions. However, some GdnHCl studies also involve dissociation of insulin oligomers. NUV CD studies with dimeric human insulin and a monomeric mutant (Asp^{B9}) show that the dimer dissociates to monomers when the GdnHCl concentration is increased from 0 to 3 M. In this GdnHCl concentration range, no effect is seen on the monomeric mutant. From 3 to 8 M GdnHCl, an identical unfolding transition is observed for both the mutant and native insulin (*28*). Similar dissociation of human insulin at low GndHCl concentrations has been demonstrated in the presence of 20% ethanol (*27*), where insulin is predominantly dimeric at the applied concentration (*25*). In another study (*24*), the GdnHCl-induced dissociation of the hexameric zinc−insulin system was studied by NUV CD, small-angle X-ray scattering (SAXS), and size-exclusion chromatography (SEC). A major transition in the 276 nm CD signal was observed over the range of 0−3.5 M GdnHCl followed by gradual changes up to 6.25 M GdnHCl. The transition represents the dissociation of hexamers via dimers to monomers and is supported by the SAXS and SEC results in the same study. The unfolding of the monomers takes place from around 3 M GdnHCl which is in accordance with ref *28*. A similar study has been performed with urea. It was found that the hexamer dissociated at low urea concentrations, and the subsequent unfolding of monomers required significantly higher urea concentrations (*36*).

It can be concluded that the mechanism of hexameric insulin denaturation is essentially dissimilar depending on whether the means of denaturation is chemical (i.e., GdnHCl or urea) or thermal. With the chemical denaturants, monomer unfolding takes place after dissociation of the hexamer. The dissociation occurs at chemical denaturant concentrations lower than that of the onset of monomer unfolding. Therefore, the unfolding takes place in the same chemical denaturant concentration range regardless of the original oligomeric state of the insulin. On the contrary, during thermal denaturation of the hexamer, dissociation takes place at temperatures well above the unfolding temperatures of monomeric insulin. In this case, the unfolding of the monomer takes place instantaneously after dissocation. Thus, in GdnHCl or urea denaturation studies, the stabilizing effect of the hexamer state does not influence the stability toward unfolding, and the monomer ΔG_u is independent of the oligomerization state at the starting point. During thermal denaturation, hexamer dissociation is the rate-limiting step and the increased stability of the hexamer state is evident. Thus, in the case of insulin hexamer denaturation by either chemical denaturants or by heating, the mechanisms are apparently fundamentally different. Also, it appears that the molecular mechanisms for chemical denaturant-induced insulin monomer unfolding and insulin hexamer dissociation are dissimilar. In the insulin hexamer with 2 zincs, each Zn^{2+} ion complexes with three His^{B10} residues and three water molecules (when no ligands other than zinc are present) (*37*, *38*). Apparently, the binding forces of zinc (complex binding) are more susceptible to disruption by chemical denaturants than the forces that maintain the protein fold (hydrogen bonding, van der Waals forces, hydrophobic interactions, and electrostatic forces). On the other hand, the zinc coordination in the hexamer has a much higher thermostability than the monomer fold.

To conclude, the complex thermal denaturation of insulin is a manifestation of oligomerization and stabilization of hexamers by zinc binding. We have shown that the biphasic thermal denaturation of the zinc−insulin system is a consequence of the coupling between insulin denaturation and zinc binding equilibria.

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