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Thermodynamic Contributions to the Stability of the Insulin Hexamer

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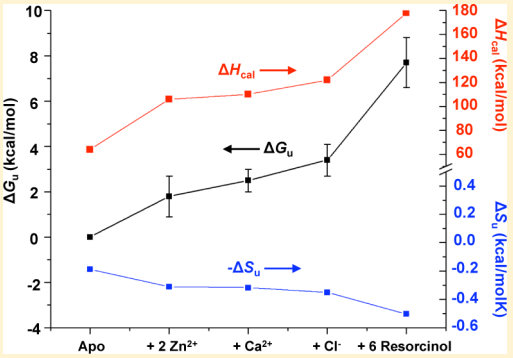
Supporting Information

ABSTRACT: The insulin hexamer is resistant to degradation and fibrillation, which makes it an important quaternary structure for its *in vivo* storage in Zn^{2+}- and Ca^{2+}-rich vesicles in the pancreas and for pharmaceutical formulations. In addition to the two Zn^{2+} ions that are required for its formation, three other species, Zn-coordinating anions (e.g., Cl^-), Ca^{2+}, and phenols (e.g., resorcinol), bind to the hexamer and affect the subunit conformation and stability. The contributions of these four species to the thermodynamics of insulin unfolding have been quantified by differential scanning calorimetry and thermal unfolding measurements to determine the extent and nature of their stabilization of the insulin hexamer. Both Zn^{2+} and resorcinol make a significant enthalpic contribution, while Ca^{2+} primarily affects the protein heat capacity (solvation) by its interactions in the central cation-binding cavity, which is modulated by the surrounding subunit conformations. Coordinating anions have a negligible effect on the stability of the hexamer, even though subunits shift to an alternate conformation when these anions bind to the Zn^{2+} ions. Finally, Zn^{2+} in excess of the two that are required to form the hexamer further stabilizes the protein by additional enthalpic contributions.



The peptide hormone insulin has been studied extensively for its physiological role in regulating blood glucose levels, its unique biochemical synthesis, its pharmacological properties in the treatment of diabetes, and its recombinant pharmaceutical preparation. Synthesized in the β cells of the pancreas where it is post-translationally cleaved into two disulfide-linked peptide chains (21-residue A and 30-residue B), insulin is stored in Zn^{2+}- and Ca^{2+}-rich vesicles as a metal-stabilized hexamer. When insulin is released into the bloodstream, the hexamer dissociates into monomers that bind to the insulin receptor.[1,2] Because the hexamer is resistant to degradation and fibrillation,[3,4] it is the preferred form of the hormone for pharmaceutical formulations.

The insulin hexamer has been structurally well characterized by X-ray crystallography.[5] It is stabilized by two Zn^{2+} ions and is best described as a trimer of dimers, with each Zn^{2+} coordinated to the B10 histidines from three of the subunits and either three waters or one coordinating anion, such as Cl^- (Figure 1).[6]

Depending on the presence of allosteric effectors, the insulin monomers in the hexamer adopt one of two conformations, *T* with its B1−B6 residues extended and a B7−B10 β turn, or *R* with its B1−B9 residues in an α helix, which exposes a hydrophobic phenol-binding pocket.[7,8] Three forms of the hexamer are stabilized by Zn^{2+}, coordinating anions (e.g., Cl^-), and phenols: T_6 (Zn^{2+}), T_3R_3 (Zn^{2+}, Cl^- or phenol), and R_6 (Zn^{2+}, Cl^-, and phenol). The B13 glutamic acids of the six subunits are clustered at the center of the hexamer and create a cation-binding cavity where one Ca^{2+} or more than two Na^+ ions and a water network are found.[9−11] Hexamers with a Ca^{2+} at this site have been shown to retain the same form,[9] which we designate with a prime (e.g., T_3R_3' is stabilized by Zn^{2+}, Cl^-, and Ca^{2+}).

In addition to its important biochemical role, the insulin hexamer is a model for metal-stabilized protein quaternary structure. While the monomer is in equilibrium with the dimer (equilibrium constant in the range of 10^5−10^6) and at higher concentrations the tetramer,[10] coordination of Zn^{2+} to the B10His residues is required to stabilize the hexamer. A number of proteins and enzymes use bridging Zn^{2+} coordination to stabilize dimer or tetramer quaternary structure, but many of these involve coordination to Cys residues.[12−14] Metal coordination has also been used to create supramolecular structures from proteins engineered to display selectively positioned metal-binding residues.[15]

Because four different types of species contribute to the stability of the insulin hexamer, it is important to determine the extent and nature of their individual contributions. Differential scanning calorimetry (DSC) and complementary thermal unfolding measurements have been used previously to study the dissociation and unfolding of the hexamer[16] and the contributions of coordinating anions and phenols to the hexamer stability.[17] An unusual biphasic thermal unfolding was reported for the Zn_2Ins_6 form; however, a simple monophasic unfolding was found with excess Zn^{2+}, and the latter condition

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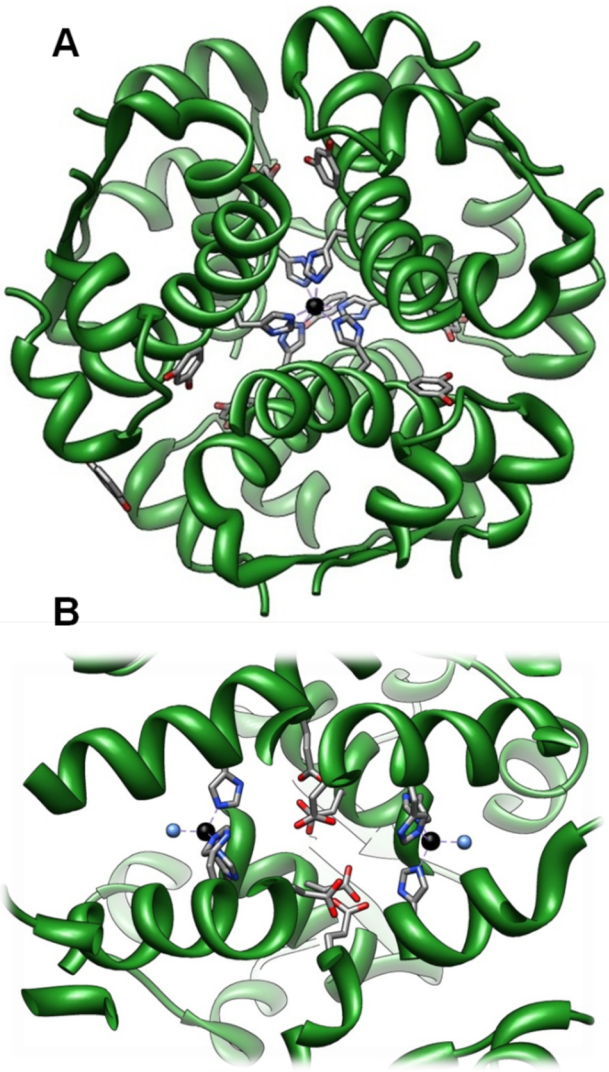

Figure 1. Structure of the R_6 insulin hexamer. (A) Viewed along the C_3 symmetry axis showing the closest Zn^{2+} (black) coordinated to three B10His residues and the bound resorcinols (gray and red). (B) Close-up after a 90° rotation, showing the bound Zn^{2+} (black) and Cl^- (blue) ions, and the B13Glu residues (gray/red). Structures created in Chimera using Protein Data Bank entry 1EVR.

was chosen for detailed investigation. Thus, many of the previously reported thermal unfolding data for insulin were obtained in the presence of excess Zn^{2+}, whose coordination and interaction with the hexamer are complex and not well understood.

In this study, we have also used DSC and thermal unfolding to investigate the stability of insulin but have focused on the well-characterized Zn_2Ins_6 hexamer form of human, porcine, and bovine insulin, which all exhibit a monophasic unfolding under our experimental conditions. This has allowed us to quantify the individual thermodynamic contributions of Zn^{2+}, Ca^{2+}, Cl^-, and resorcinol to the stability of the insulin hexamer and obtain new insight into this metal-stabilized quaternary structure.

MATERIALS AND METHODS

Bovine (pancreas, catalog no. I5500), porcine (pancreas, catalog no. I5523), and human (recombinant from yeast, catalog no. I2643) insulins were purchased from Sigma-Aldrich, as were other analytical grade chemicals. Humalog (Insulin Lispro, Eli Lilly) was obtained from a local pharmacy as a 10 mL solution containing this fast-acting insulin, which was purified as described below. Nanopure (18 MΩ) water was obtained with a Millipore water purification system and used for all solutions. Buffer solutions were treated with Chelex 100 resin to remove trace metals and then filtered through a 0.22 μm filter prior to use.

Insulin samples were prepared as follows. A 5 mg sample of native insulin was dissolved in 2 mL of Nanopure water, and the pH was adjusted to ~3. This solution was passed through a 28 cm × 0.5 cm Chelex 100 column to remove metal cations, and the absence of residual Zn^{2+} and Ca^{2+} was confirmed by ICP-MS analysis at the Dartmouth Trace Element Analysis Core Laboratory. The identity and purity of the insulin were confirmed by a single band on SDS−PAGE and a single 5.8 kDa peak in the MALDI-TOF mass spectrum (Figure S1 of the Supporting Information), obtained at the Dartmouth Molecular Biology Core Laboratory. The protein was transferred into the desired buffer solution using a 3.5 kDa Amicon centrifuge filter. The insulin concentration was determined by the absorbance at 276 nm with an ε_{276} of 6200 M^{-1} cm^{-1}[18] and is given per monomer, unless indicated otherwise. Insulin hexamer samples were prepared by the addition of appropriate amounts of Zn^{2+}, Ca^{2+}, Cl^-, and resorcinol, followed by incubation for at least 2 h at 4 °C.

DSC data were obtained on insulin samples in 50 mM Tris buffer (pH 7.4) with a MicroCal (Northampton, MA) VP-DSC instrument. Typical scans covered the 15−100 °C range at a scan rate of 80 °C/h. Reference scans were obtained on the 50 mM Tris buffer (pH 7.4) solution, and these were subtracted from experimental scans prior to concentration normalization. Data analysis was conducted with Origin 7.0 (OriginLab, Northampton, MA), as described in detail elsewhere.[19,20]

Thermal denaturation was monitored by circular dichroism (CD) using a JASCO (Easton, MD) J-815 CD spectrometer and a variable-temperature Peltier cell. Samples were dissolved in 10 mM sodium phosphate buffer (pH 7.4), and the ellipticity at 220 nm, due to the α helical structure of insulin, was monitored over the temperature range of 20−100 °C at a rate of 1 °C/min. Thermodynamic parameters were determined through nonlinear curve fitting using the MATLAB (MathWorks, Natick, MA) curve fitting tool. Data were fit to eq 1

$$f(X) = \frac{(m_f X + b_f + m_u X + b_u)\exp^{\left[\frac{-\Delta H_{vH}}{R}\left(\frac{1}{X}-\frac{1}{T_m}\right)\right]}}{1+\exp^{\left[\frac{-\Delta H_{vH}}{R}\left(\frac{1}{X}-\frac{1}{T_m}\right)\right]}} \quad (1)$$

where m_f and b_f represent the slope and y-intercept, respectively, of the folded (low-temperature) regions of the scan, m_u and b_u represent the slope and y-intercept, respectively, of the unfolded (high-temperature) regions of the scan, R is the gas constant (8.314 J/mol K), ΔH_{vH} is the enthalpy of unfolding, and T_m is the melting temperature.

RESULTS

The thermal stability of human insulin in the absence of metal ions (apo) and in the presence of 0.33 Zn^{2+} per monomer (two per hexamer), along with other species (Ca^{2+}, Cl^-, and resorcinol), was measured via DSC. Figure 2 shows representative endotherms for each of the hexamer forms.

These thermal scans exhibit a single broad envelope (at least two Gaussians) with a well-defined T_m, a clear baseline shift (ΔC_p), and no obvious signs of aggregation (precipitous drop

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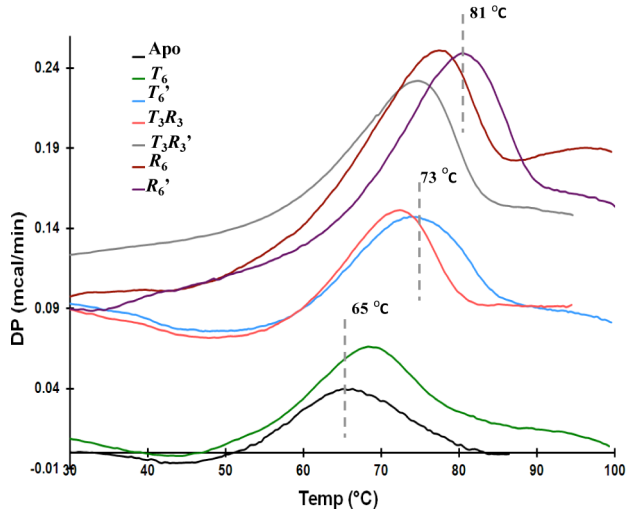

Figure 2. Representative raw DSC endotherms (offset for the sake of clarity) of 0.10 mM human insulin with the indicated hexamer structure in 50 mM Tris buffer (pH 7.4): T_6 (33 μM $ZnSO_4$), T_6' (33 μM $ZnSO_4$ and 33 μM $CaSO_4$), T_3R_3 (33 μM $ZnCl_2$), T_3R_3' (33 μM $ZnCl_2$ and 33 μM $CaCl_2$), R_6 (33 μM $ZnCl_2$ and 5 mM resorcinol), and R_6' (33 μM $ZnCl_2$, 33 μM $CaCl_2$, and 5 mM resorcinol).

in C_p when $T > T_m$).[21] The value of ΔH_{cal} was determined from integration of the whole envelope.

Certain experimental parameters, especially reversibility and scan rate, are crucial for drawing thermodynamic insight from these data. As reported previously for apo insulin and insulin with excess Zn^{2+} (five per hexamer),[16] unfolding of the T_6 form was found to be quasi-reversible (Figure S2 of the Supporting Information), losing 5−25% of the enthalpy in the second scan, depending on how far the initial scan extends beyond T_m. However, the extent of the scan and percent reversibility had a negligible impact on T_m and ΔH_{cal} values determined from the first scan. The dependence of the DSC data on the scan rate in the 10−90 °C/h range was also investigated previously for apo insulin and insulin with excess Zn^{2+}.[16] Only at rates of <30 °C/h were significant differences noted. However, Wollmer and co-workers have shown that subunit exchange is slowed significantly by phenol at 20 °C.[22] Because this could lead to nonequilibrium conditions during unfolding, the scan rate dependence of the DSC data of R_6' was investigated over the range of 45−80 °C (Figure S3 of the Supporting Information). A negligible effect on T_m and ΔH_{cal} is found, although a shift in the unfolding envelope suggests there is a kinetic barrier for a lower-temperature component of the overall unfolding process.

Finally, DSC measurements with porcine insulin revealed a buffer dependence to the unfolding thermodynamics (Table S1 of the Supporting Information), which may relate to interaction of the buffer with the protein, the Zn^{2+} ions, and/or protons from (de)protonation(s) coupled with unfolding. Investigating the source of this variability was beyond the scope of this study, and Tris buffer was used for all DSC data reported herein.

Table 1 contains the average experimental values obtained from these DSC data. As indicated by the T_m values, all of the hexamer forms are more stable than the apo protein, which is primarily a dimer in solution at these concentrations (Figure S1 of the Supporting Information).[10] The addition of Ca^{2+} imparts greater stability to the hexamer (T_6' vs T_6), and resorcinol stabilizes the hexamer even further (R_6 vs T_3R_3). The two Zn^{2+} ions provide significant enthalpic stabilization, which is expected from their coordination to the B10His residues of three subunits.

The additional stabilization afforded by Ca^{2+} is not found in the unfolding enthalpy but does correlate with an increase in ΔC_p, suggesting an entropic origin, as found with binding of Ca^{2+} to EDTA.[23,24] Resorcinol provides nearly 10 kcal/mol of enthalpic stabilization, likely originating from molecular interactions (hydrogen bonding and π-stacking) at the phenol-binding site of each insulin subunit. These data show only a small thermodynamic contribution from the protein conformational switch from T to R, as indicated by similar T_m and ΔH_{cal} values for T_6 and T_3R_3 and for T_6' and T_3R_3'.

For comparison to the values determined by DSC, thermal unfolding was also quantified by the temperature dependence of CD features originating from the insulin secondary structure (Figure S4 of the Supporting Information).[4,16] Representative unfolding data for T_6 insulin are shown in Figure 3, which reveals a gradual loss of secondary structure measured at 220 nm.

Unfolding curves for the different hexamer forms monitored at 220 nM (Figure S5 of the Supporting Information) show an increase in the temperature of the inflection, which parallels the increase in T_m found by DSC. The broad temperature range over which the protein secondary structure is lost is consistent with the DSC data, which have broad unfolding peaks characteristic of a non-two-state unfolding process.[16] Best fits of the data to eq 1 were used to extract the unfolding transition temperatures and enthalpies of several of the hexamer forms, which are also included in Table 1. The trend is the same with both methods, although the melting temperature determined by CD is consistently 2−3 °C higher than that found by DSC. This difference likely results from the different buffers or the

Table 1. Average Thermal Unfolding Values for the Indicated Hexamer Form of Human Insulin Determined from DSC Measurements in 50 mM Tris Buffer (pH 7.4) and Thermal Unfolding Monitored by CD in 10 mM Phosphate Buffer (pH 7.4)

	DSC			CD	
hexamer	T_m (°C)	$\Delta H_{cal}{}^a$ (kcal/mol)	$\Delta C_p{}^a$ (kcal mol^{-1} K^{-1})	$T_m{}^b$ (°C)	$\Delta H_{vH}{}^{a,b}$ (kcal/mol)
apo	65 ± 0.5	11 ± 1	0.2 ± 0.3	67 ± 0.6	10 ± 1
T_6	68 ± 0.9	18 ± 1	0.4 ± 0.2	71 ± 0.3	16 ± 1
T_6'	73 ± 0.2	19 ± 1	0.9 ± 0.4	74	24
T_3R_3	71 ± 0.7	18 ± 1	0.6 ± 0.3	74 ± 0.4	22 ± 1
T_3R_3'	75 ± 0.5	21 ± 1	1.0 ± 0.1	79	25
R_6	79 ± 0.8	27 ± 1	0.8 ± 0.2	–	–
R_6'	81 ± 1	30 ± 1	1.1 ± 0.3	84	33

[a]Determined on a per monomer basis. [b]Calculated with eq 1.

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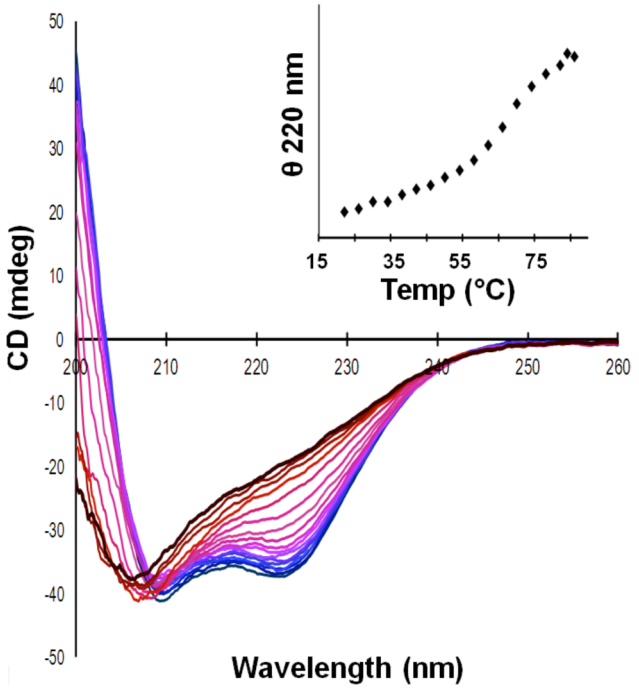

Figure 3. Representative thermal unfolding of the T_6 human insulin hexamer monitored by CD over the temperature range of 20−90 °C (from blue to dark red) in 10 mM phosphate buffer (pH 7.4). The inset is a plot of ellipticity at 220 nm, which can be fit to eq 1 (T_m = 67.5 °C, and ΔH_{vH} = 106.3 kcal/mol of hexamer).

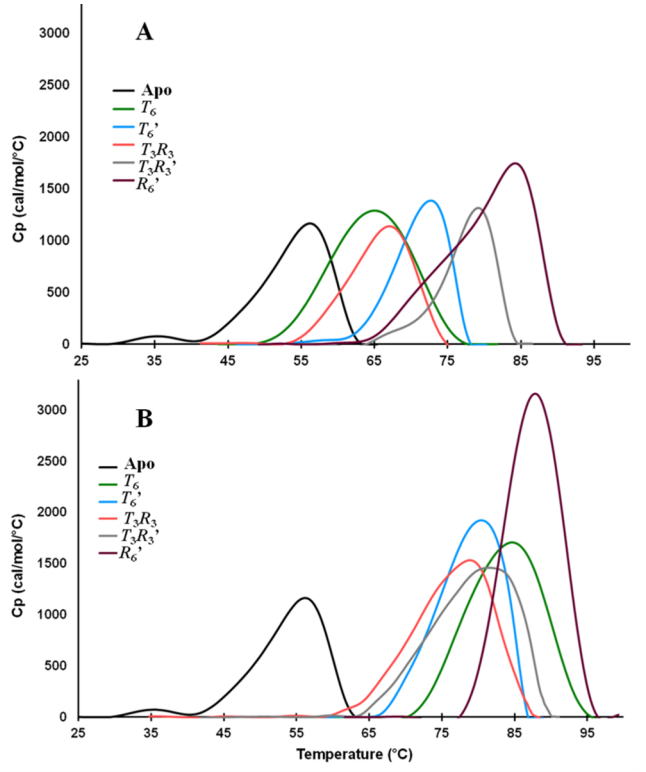

Figure 4. Representative baseline-adjusted, concentration-normalized DSC endotherms of 0.10 mM bovine insulin with the indicated hexamer structure in 50 mM Tris buffer (pH 7.4). (A) Two Zn^{2+} ions per hexamer (concentrations of stabilizing species are given in the legend of Figure 2) and (B) 9.6 Zn^{2+} ions per hexamer: T_6 (0.16 mM $ZnSO_4$), T_6' (0.16 mM $ZnSO_4$ and 0.16 mM $CaSO_4$), T_3R_3 (0.16 mM $ZnCl_2$), T_3R_3' (0.16 mM $ZnCl_2$ and 0.16 mM $CaCl_2$), and R_6' (0.16 mM $ZnCl_2$, 0.16 mM $CaCl_2$, and 5 mM resorcinol).

nature of the measured property (enthalpy vs secondary structure).

Unfolding calorimetric measurements were also taken on porcine and bovine insulin, which differ from human insulin by one amino acid and three amino acids, respectively, and adopt similar hexameric structures upon Zn^{2+} coordination. The baseline-corrected DSC endotherms of apo bovine insulin and its hexamer forms with two Zn^{2+} ions per hexamer are shown in Figure 4A.

These data are qualitatively similar to those of human insulin, with an increase in stability upon addition of Zn^{2+}, Ca^{2+}, and resorcinol. In addition to these results with stoichiometric Zn^{2+} (two per hexamer), the effect of excess Zn^{2+} (9.6 per hexamer) on thermal unfolding is shown in Figure 4B. All hexamer endotherms are now shifted to higher temperatures, and contributions from the excess Zn^{2+} mask the stabilizing contributions of other species. Table 2 contains the experimental values determined from DSC measurements on bovine insulin with stoichiometric and excess Zn^{2+}.

While there are quantitative differences between bovine and human insulin, most notably the more stable (higher T_m) human apo protein and the generally larger unfolding enthalpies of the human hexamers, the two insulins are qualitatively similar.

DISCUSSION

The insulin monomer is susceptible to degradation and fibrillation,[25−27] while the Zn-stabilized hexamer is much less so, making this quaternary structure important for storage of the peptide hormone in pancreatic vesicles, as well as pharmaceutical preparations. Upon being released, or delivered, into the bloodstream, where Zn^{2+} levels are low, the hexamer dissociates into monomers that are competent to bind the insulin receptor. The Zn^{2+} coordination in the hexamer has been characterized by X-ray crystallography (Figure 1), which also revealed two conformations of the subunits, *T* and *R*, that are determined by anions binding to the Zn^{2+} and/or phenols binding in hydrophobic pockets of the protein;[6,28,29] coordinating anions bind to one Zn^{2+} and shift T_6 to T_3R_3, while phenols bind to each subunit and further shift T_3R_3 to R_6.[6,8,30] Because the stability of the insulin hexamer is predominantly due to thermodynamic contributions of the metal ions and allosteric factors, we have used primarily DSC to determine the nature and extent to which they stabilize this quaternary structure.

A previous study also used DSC to investigate Zn^{2+} stabilization of human insulin and examined the scan rate dependence and reversibility of thermal unfolding in the absence and presence of excess Zn^{2+}.[16] We have shown here (Figures S2 and S3 of the Supporting Information) the quasi-reversibility of Zn_2Ins_6 (T_6) unfolding and the absence of a significant kinetic barrier to thermal unfolding of the most stable Zn_2Ins_6 hexamer (R_6'). The previous study also reported an unusual, though not unprecedented,[31] biphasic endotherm for insulin samples with two Zn^{2+} ions per hexamer, but a single unfolding transition at a somewhat higher temperature with excess Zn^{2+} (five to six per hexamer). This latter sample was then studied in more detail.

Our DSC data for human insulin differ from these previous results in one important respect. Under similar pH, buffer, and ionic strength conditions, we find T_m and ΔH_{cal} values that are

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Table 2. Average DSC Values for the Indicated Hexamer Forms of Bovine Insulin in 50 mM Tris Buffer (pH 7.4)

	2 Zn^{2+} ions per hexamer			9.6 Zn^{2+} ions per hexamer		
hexamer	T_m (°C)	ΔH_{cal}[a] (kcal/mol)	ΔC_p[a] (kcal mol^{-1} K^{-1})	T_m (°C)	ΔH_{cal}[a] (kcal/mol)	ΔC_p[a] (kcal mol^{-1} K^{-1})
apo	59 ± 3	12 ± 3	0.4 ± 0.3	59 ± 3	12 ± 3	0.4 ± 0.3
T_6	67 ± 2	14 ± 3	0.6 ± 0.3	82 ± 2	27 ± 4	1.0 ± 0.6
T_6'	74 ± 2	17 ± 2	1.0 ± 0.2	82 ± 1	27 ± 3	1.0 ± 0.2
T_3R_3	67 ± 1	16 ± 4	0.5 ± 0.1	79 ± 1	22 ± 2	1.2 ± 1
T_3R_3'	77 ± 3	16 ± 1	1.1 ± 0.2	80 ± 2	20 ± 4	1.0 ± 0.1
R_6'	83 ± 1	26 ± 2	1.1 ± 0.4	84 ± 2	26 ± 4	0.9 ± 0.4

[a]Determined on a per monomer basis.

similar to those reported earlier for apo insulin and samples with excess Zn^{2+} (Table 3).

Table 3. Experimental DSC Parameters of Apo and T_6 Human Insulin with the Indicated Zn^{2+} Stoichiometry

	apo		0.33 Zn^{2+} per monomer		≥1 Zn^{2+} per monomer	
source	T_m (°C)	ΔH_{cal}[c] (kcal/mol)	T_m (°C)	ΔH_{cal}[c] (kcal/mol)	T_m (°C)	ΔH_{cal}[c] (kcal/mol)
ref 16[a]	68.5	24	72, 86	–	87.6	34.5
this work[b]	67	23	74	27	83	31

[a]600 μM insulin in 7 mM phosphate buffer (pH 7.4). [b]100 μM insulin in 10 mM phosphate buffer (pH 7.4). [c]Determined on a per monomer basis.

However, we find only a single unfolding transition for Zn_2Ins_6 hexamer samples, in contrast to the reported biphasic endotherm. This result is consistently found in different buffers (Figure S6 of the Supporting Information). The lower of the two reported T_m values is similar to our value for Zn_2Ins_6, while the higher T_m value matches that found with excess Zn^{2+} (Table 3). On the basis of a number of analytical results, the purity and preparation of our insulin appear to be comparable to those used in the earlier study. A significant difference, however, is the considerably higher concentration of insulin (600 μM) used for the previously reported DSC measurements. The quaternary structure of apo insulin depends on its concentration, with predominantly dimers at 100 μM (our concentration) but a significant fraction of tetramers at 600 μM.[10] This higher degree of oligomerization of apo insulin may alter the thermal unfolding of concentrated samples with 0.33 Zn^{2+} per monomer, resulting in the reported second transition that correlates with the unfolding of insulin in the presence of excess Zn^{2+}. This higher-temperature transition was attributed to redistribution of the Zn^{2+} during thermal unfolding,[16] but this would appear to require higher protein concentrations, as we find no evidence for this phenomenon in our data.

In a subsequent study by the same group,[17] the effect of the allosteric species, coordinating anions and phenols, on the thermal stability of insulin was investigated. On the basis of their previous results, this study examined the binding of these species to insulin hexamers with excess Zn^{2+} (~5 per hexamer). We have also considered the effect of excess Zn^{2+} on the thermal stability of insulin with data collected on the bovine protein (Figure 4). Excess Zn^{2+} leads to an ~15 °C increase in T_m and a near doubling of ΔH_{cal} for the T_6 hexamer, and now all hexamer forms have similar unfolding thermodynamics (Table 2). Similar results were reported, and we have confirmed (Table S2 of the Supporting Information), for human insulin with excess Zn^{2+}, although samples with phenol have a noticeably higher enthalpy of unfolding.[17] Thus, we and others find that extra stabilization is imparted by Zn^{2+} ions in excess of the two that stabilize the hexamer. However, insulin samples with excess Zn^{2+} are not as structurally or chemically well characterized as the Zn_2Ins_6 hexamer, which was the focus of this study.

To quantify the contributions of Zn^{2+}, Cl^-, Ca^{2+}, and resorcinol to the stability of the insulin hexamer, thermal unfolding values for the Zn-stabilized hexamer and the hexamer in the presence of these additional species are compared to values for insulin in the absence of Zn^{2+} (apo). Insulin exists primarily as a dimer at the concentrations used in this study, so comparison to the hexamer necessarily involves a contribution from protein dimerization and possibly more aggregated structures. To evaluate this contribution, DSC measurements were made on a commercially available fast-acting insulin (Humalog)[32] that swaps the positions of two residues (B28,B29, Pro-Lys → Lys-Pro) to suppress dimerization. As shown in Figure 5, which compares DSC data for native insulin

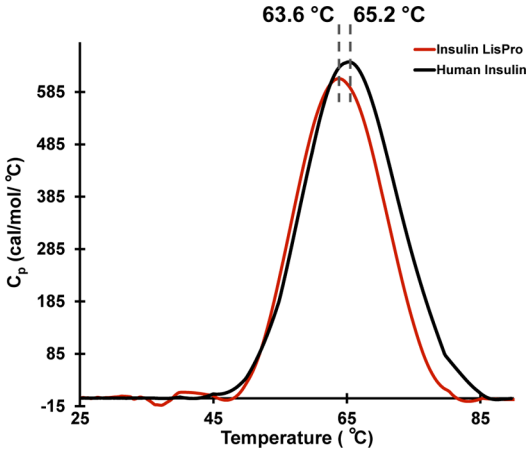

Figure 5. Representative baseline-adjusted, concentration-normalized DSC endotherms of 0.10 mM human insulin (black) and Humalog (Insulin LisPro) (red) in 50 mM Tris buffer (pH 7.4).

and this monomeric insulin, the T_m of the monomer form (64 ± 0.8 °C) is indistinguishable from that of the native protein (65 ± 0.5 °C) and its unfolding enthalpy (9 ± 0.5 kcal/mol) is only slightly lower than that of native insulin (11 ± 1 kcal/mol).

A previous study[16] also investigated the dimer contribution to insulin thermal stability by comparing DSC results for native insulin to those of two other forms that are modified to suppress dimerization. These monomer forms have quantitatively higher T_m and lower ΔH_{cal} values, and the unfolding is

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Table 4. Thermodynamic Values for the Unfolding of Human Insulin Hexamers Determined by DSC on a per Hexamer Basis

hexamer	$\Delta G_u^{a,b}$ (kcal/mol)	$\Delta G_u^{a,c}$ (kcal/mol)	ΔH_{cal} (kcal/mol)	ΔH_{vH}^{d} (kcal/mol)	ΔS_u^{e} (cal mol^{-1} K^{-1})
apo	0	5.6 ± 0.6	64 ± 1	60 ± 6	189 ± 3
T_6	1.8 ± 1.0	11.1 ± 0.9	106 ± 2	96 ± 6	311 ± 6
T_6'	2.5 ± 0.5	12.5 ± 0.6	110 ± 2	144	318 ± 6
T_3R_3	1.8 ± 0.9	11.7 ± 0.8	107 ± 2	132 ± 6	311 ± 6
T_3R_3'	3.4 ± 0.7	14.5 ± 0.7	122 ± 2	150	351 ± 6
R_6	6.2 ± 0.9	21.4 ± 0.9	162 ± 2		460 ± 6
R_6'	7.7 ± 1.1	24.4 ± 1.0	178 ± 2	198	503 ± 6

[a]Calculated with eq 2. [b]At a reference temperature of 338 K (apo T_m). [c]At a reference temperature of 298 K. [d]Calculated from CD data with eq 1. [e]Determined at T_m by $\Delta H_{cal}/T_m$.

noticeably broader. However, in contrast to the monomer studied here, these introduce one negative charge (B28, Pro → Asp) and two negative charges (B9, Ser → Asp; B27, Thr → Glu), which appear to quantitatively affect the insulin thermal unfolding thermodynamics. Thus, measurements on an insulin that is conservatively modified to suppress dimer formation indicate that the dimerization, and possible contributions from more aggregated forms, contributes at most a few kilocalories per mole to the unfolding enthalpy measured by DSC. This suggests that insulin dimerization is predominantly an entropy-driven process.

The stability of the protein structure can also be investigated by the effect of chaotropic species (GuHCl or urea) on the secondary, tertiary, and quaternary protein structure. Fink and co-workers have provided such insight in the course of their studies of insulin fibrillation.[4] Using several analytical and spectroscopic methods on insulin samples containing Zn^{2+} and thus hexamer quaternary structure, they showed that low concentrations (~0.5 M) of GuHCl dissociate the hexamer and that higher concentrations are needed to unfold the insulin subunits. We have used CD spectroscopy to monitor GuHCl unfolding of the various hexamer forms (Figure S7 of the Supporting Information), and our results are consistent with those reported by Fink and co-workers. Because Zn^{2+} coordination is disrupted by guanidine, no additional stabilization is provided by the metal to unfolding of the protein by chaotropic agents. This fundamentally different unfolding mechanism prevents comparison between thermodynamic values obtained by temperature-dependent and chaotrope-dependent measurements.

The goal of this study was to determine the individual thermodynamic contributions of Zn^{2+}, Ca^{2+}, coordinating anions (e.g., Cl^-), and phenols (e.g., resorcinol) to the stability of the Zn_2Ins_6 insulin hexamer. Experimental values from DSC measurements can be used to determine the free energy of unfolding, ΔG_u. Because this analysis (eq 2) requires a reference temperature[33]

$$\Delta G(T) = \Delta H_{cal}\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left[(T - T_m) - T\ln\left(\frac{T}{T_m}\right)\right] \quad (2)$$

these values have been determined both with the T_m of the apo form (338 K) and with the standard value of 298 K. Values of ΔS_u were determined at the T_m of each sample, where $\Delta G_u = 0$ and $\Delta S_u = \Delta H_{cal}/T_m$. Table 4 summarizes the unfolding thermodynamics of the different hexamer forms of human insulin.

Using the thermodynamic data in Table 4, we are now able to determine the change in these values associated with each of the species that binds to the protein and thus their contribution to its thermal stability. Table 5 indicates the contributions of Zn^{2+}, Ca^{2}, Cl^-, and resorcinol to the unfolding thermodynamics, and Figure 6 shows a graphical presentation of the thermodynamic contributions from successive addition of these species to insulin.

Table 5. Individual Thermodynamic Contributions to the Thermal Stability of the Human Insulin Hexamer Determined on a per Hexamer Basis

species	$\Delta\Delta G_u^{a}$ (kcal/mol)	$\Delta\Delta H_{cal}$ (kcal/mol)	$\Delta\Delta S_u$ (cal mol^{-1} K^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})
Zn^{2+}	1.8 ± 1.0	42 ± 2	122 ± 7	0.4 ± 0.2
Ca^{2+}	1.2 ± 1.0	12 ± 3	40 ± 9	0.7 ± 0.3
Cl^-	0.5 ± 1.1	7 ± 3	17 ± 8	0.2 ± 0.3
resorcinol	4.3 ± 1.2	56 ± 3	150 ± 9	0.4 ± 0.2

[a]At a reference temperature of 338 K.

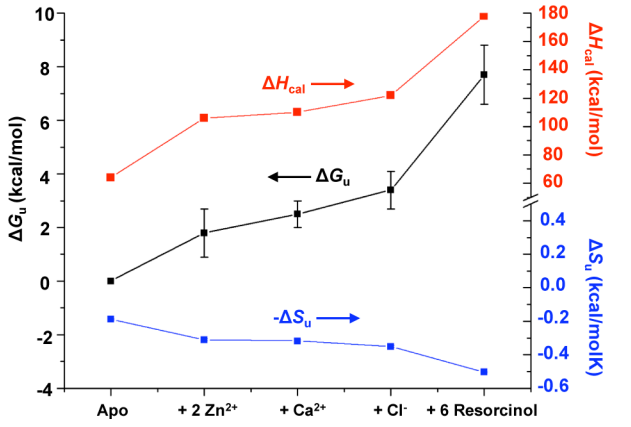

Figure 6. Plot of the enthalpic (ΔH_{cal}) and entropic (ΔS_u) contributions to the free energy (ΔG_u) of unfolding of human insulin from sequential addition of Zn^{2+}, Ca^{2+}, Cl^-, and resorcinol. Error bars for apo ΔG_u and for all ΔH_{cal} and ΔS_u values are smaller than the symbols.

Their effect on the overall stability, which is seen experimentally in ΔT_m and quantified as $\Delta\Delta G_u$ of unfolding, is the balance between their contributions to the unfolding enthalpy, $\Delta\Delta H_{cal}$, and the unfolding entropy, $\Delta\Delta S_u$.

Because Zn^{2+} is required to form the hexamer, its contributions include both those from its coordination to the protein and the intersubunit interactions of three insulin dimers. (Note that data for the apo sample quantify the unfolding of the insulin dimer.) Because the Zn–His bond enthalpy is ~5 kcal/mol,[34] up to ~30 kcal/mol of the stabilization enthalpy may be associated with metal coordina-

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Table 6. Thermodynamic Contributions of Ca^{2+} to Hexamer Stability (T_6'-T_6) and Contributions of Ca^{2+} in the Presence of Cl^- (T_3R_3'-T_3R_3), and in the Presence of Cl^- and Resorcinol (R_6'-R_6) on a per Hexamer Basis

	$\Delta\Delta G_u$[a] (kcal/mol)	$\Delta\Delta G_u$[b] (kcal/mol)	$\Delta\Delta H_{cal}$ (kcal/mol)	$\Delta\Delta S_u$ (cal mol^{-1} K^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})
(T_6'-T_6)	0.7 ± 0.5	1.4 ± 0.7	4 ± 2	7 ± 7	0.5 ± 0.2
(T_3R_3'-T_3R_3)	1.6 ± 0.7	2.8 ± 0.9	15 ± 3	40 ± 7	0.8 ± 0.2
(R_6'-R_6)	1.5 ± 0.8	3.0 ± 1.0	16 ± 3	43 ± 7	0.9 ± 0.2

[a]At a reference temperature of 338 K. [b]At a reference temperature of 298 K.

Table 7. Individual Thermodynamic Contributions to the Thermal Stability of the Bovine Insulin Hexamer (2 Zn^{2+} ions per hexamer) on a per Hexamer Basis

species	$\Delta\Delta G_u$[a] (kcal/mol)	$\Delta\Delta H_{cal}$ (kcal/mol)	$\Delta\Delta S_u$ (cal mol^{-1} K^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})
Zn^{2+}	1.8 ± 2.0	12 ± 4	28 ± 11	0.3 ± 0.3
Ca^{2+}	2.2 ± 2.5	9 ± 4	22 ± 10	0.7 ± 0.4
Cl^-	0.4 ± 2.5	4 ± 4	11 ± 10	0.2 ± 0.4
resorcinol	5.1 ± 3.0	59 ± 4	161 ± 10	0.3 ± 0.2

[a]At a reference temperature of 332 K.

Table 8. Individual Thermodynamic Contributions to the Thermal Stability of the Bovine Insulin Hexamer (9.6 Zn^{2+} ions per hexamer) on a per Hexamer Basis

species	$\Delta\Delta G_u$[a] (kcal/mol)	$\Delta\Delta H_{cal}$ (kcal/mol)	$\Delta\Delta S_u$ (cal mol^{-1} K^{-1})	$\Delta\Delta C_p$ (kcal mol^{-1} K^{-1})
Zn^{2+}	9.0 ± 1.9	89 ± 5	234 ± 13	1.1 ± 0.7
Ca^{2+}	−0.2 ± 2.1	5 ± 5	−15 ± 12	0.2 ± 0.4
Cl^-	−2.7 ± 1.9	−34 ± 5	−94 ± 12	−0.1 ± 0.5
resorcinol	3.3 ± 2.7	35 ± 5	94 ± 13	0.2 ± 0.4

[a]At a reference temperature of 332 K.

tion, with the remaining ≥10 kcal/mol due to contacts at the interfaces between the dimers of subunits. The $\Delta\Delta S_u$ value originates from dissociation of the hexamer and loss of the Zn^{2+} ions. Because it has been shown that two to three protons bind to insulin upon formation of the hexamer at pH 7.4,[35] they would also be lost upon its dissociation. However, this is countered by the solvation of the unfolded protein, which may be substantial for the insulin hexamer because of fewer bound waters relative to other proteins on a per residue basis.[5]

While Ca^{2+} leads to a noticeable shift in the T_m, its impact on ΔG_u is small and the enthalpic and entropic factors associated with its binding in the B13Glu cavity are not large. However, the effect of Ca^{2+} on ΔC_p is nearly twice as large as that of other species. This may be due to its effect on the hydrogen bonding and water network in the central cation-binding cavity.

The representative coordinating anion Cl^- binds to one of the Zn^{2+} ions and shifts the conformation of its three subunits from *T* to *R*, with its additional α helical secondary structure. The data in Table 5 show that this contributes very little to the protein unfolding thermodynamics. As indicated by CD measurements (Figure 3), secondary structure is lost upon thermal unfolding. Therefore, the loss of the additional helix from subunits in the *R* conformation, and possibly cleavage of the Zn−Cl bond, either is a negligible contribution to the unfolding thermodynamics or, more likely, is compensated by other changes in the *R* subunit unfolding and/or Zn^{2+} coordination.

As seen in Table 5 and Figure 6, resorcinol makes a significant contribution to the stability of the insulin hexamer, as has been shown previously.[30,36,37] Although its binding shifts the three remaining subunits to the *R* conformation, this is expected to have a negligible thermodynamic impact on the unfolding, as indicated above. Therefore, the large enthalpic stabilization associated with resorcinol is primarily due to its binding to the subunits, which is ∼10 kcal/mol of phenol. The large, but incompletely compensating, entropic contribution to the unfolding must be due to loss of the six phenols per hexamer. The thermodynamics of binding of phenols to the insulin hexamer have been studied by isothermal titration calorimetry (ITC) and shown to involve considerable enthalpy.[38,39] However, the binding thermodynamics are complex because of interactions among the subunits,[39] and direct comparisons between the reported ITC results and our DSC results are not clear.

It was initially assumed that thermodynamic contributions of Ca^{2+}, Cl^-, and resorcinol to hexamer stability were additive. The data in Table 4 now allow this assumption to be tested for the case of Ca^{2+}. Table 6 shows the additional stabilization of Ca^{2+} alone and in the presence of Cl^- and resorcinol.

While errors associated with the free energies are large relative to $\Delta\Delta G_u$, the directly measured stabilization enthalpy ($\Delta\Delta H_{cal}$) and entropy ($\Delta\Delta S_u$) values indicate that Ca^{2+} has a larger effect when at least three of the subunits are in the *R* conformation. Thus, the structure of the surrounding protein subunits has a thermodynamic impact on the central cation-binding cavity.

The unfolding thermodynamics of the bovine insulin hexamer (Table S3 of the Supporting Information) have been used to determine the individual contributions of Zn^{2+}, Ca^{2+}, Cl^-, and resorcinol to the thermal stability of this protein. Table 7 contains these data for comparison to the human insulin data listed in Table 5.

Although the errors in $\Delta\Delta G_u$ are somewhat larger, the results are qualitatively similar, particularly for the phenol thermodynamics. The major difference is the smaller enthalpy and entropy associated with Zn^{2+} stabilizing the hexamer, although the overall stabilization appears to be similar. The few residues that differ between human and bovine insulin are found in the

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A8−A10 loop, which contributes to the conformational flexibility of the insulin monomer and is manifested in thermodynamic differences between the species.[40]

The unfolding thermodynamics of bovine insulin in the presences of excess Zn^{2+} (Table S4 of the Supporting Information) allows us to determine the contributions of the individual species under these conditions (Table 8).

As is obvious from the T_m values (Figure 4B and Table 2), additional Zn^{2+} significantly enhances the thermal stability. Relative to the case with stoichiometric Zn^{2+} (Table 7), the enthalpic contribution of the Zn^{2+} increases almost 8-fold, and only resorcinol contributes any additional stabilization when excess Zn^{2+} is present. Now Cl^- has a slightly destabilizing effect that may be due to its competitive binding to the extra stabilizing Zn^{2+}.

The thermodynamic contributions of quaternary structure to protein thermal stability have been considered theoretically[41,42] and investigated, along with allosteric factors, for several proteins. Although hemoglobin has not been studied in this regard to the extent that one would expect,[43,44] aspartate transcarbamoylase (ATCase) certainly has.[45−47] The active form of this enzyme consists of two catalytic (C) trimers and three regulatory (R) dimers assembled into a C_6R_6 structure exhibiting two unfolding events (T_{m1} = 72.5 °C, and ΔH_{cal1} = 146 ± 10 kcal/mol; T_{m2} = 82 °C, and ΔH_{cal2} = 543 ± 18 kcal/mol) that differ from those of the isolated R_2 (T_m = 55 °C, and ΔH_{cal} = 65 ± 1 kcal/mol) and C_3 (T_m = 80 °C, and ΔH_{cal} = 395 ± 10 kcal/mol) subunits,[45] which reflects the thermodynamic consequences of intersubunit interactions and changes in the secondary and tertiary structure of the subunits in the dodecamer, as indicated by CD measurements.[48] Binding of the bisubstrate analogue *N*-(phosphonoacetyl)-L-aspartate (PALA) to the C subunits of C_6R_6 stabilizes both unfolding events, indicating an intersubunit interaction with the R subunits. In contrast, the activator ATP or the inhibitor CTP causes similar shifts in the unfolding events of C_6R_6 as found upon their interaction with the isolated subunits, indicating they have a localized effect on the subunits in the dodecamer. Although the ATCase quaternary structure is more complex than that of insulin, comparison of the overall contributions of PALA to the thermal stability of the ATCase dodecamer (ΔT_m = 7.5 °C, and $\Delta\Delta H_{cal}$ = 114 ± 15 kcal/mol)[45] to the contributions of resorcinol to the thermal stability of the insulin hexamer (ΔT_m = 7 ± 1 °C, and $\Delta\Delta H_{cal}$ = 55 ± 2 kcal/mol) reveals a similar overall stabilization and a stabilization enthalpy for the hexamer that is approximately half that of the dodecamer.

In summary, this study has quantified the thermodynamics of the thermal stabilization provided by Zn^{2+} coordination and assembly of the insulin hexamer, as well as that provided by the allosteric species Ca^{2+}, Cl^-, and resorcinol. We find typical non-two-state thermal unfolding of the Zn_2Ins_6 hexamer, in contrast to biphasic unfolding reported earlier with higher protein concentrations. Enthalpic contributions from Zn^{2+} coordination and resorcinol binding provide the largest stabilization of the protein. While the stabilization by Ca^{2+} is modest, its contributions are modulated by the conformation of the insulin subunits that create the cation-binding cavity in the hexamer. Finally, the additional thermal stability provided by excess Zn^{2+} ions beyond the two that are required to form the hexamer has been quantified.

■ ASSOCIATED CONTENT

Supporting Information

Mass spectral characterization, additional DSC data and tables of thermodynamic values, CD spectra and fits of their temperature dependence, experimental procedure, and fits of GuHCl unfolding monitored by CD. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

DSC, differential scanning calorimetry; ICP-MS, inductively coupled plasma mass spectrometry; CD, circular dichroism; GuHCl, guanidine hydrochloride; ITC, isothermal titration calorimetry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PALA, *N*-(phosphonoacetyl)-L-aspartate.

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