Supporting Information

Thermodynamic Contributions to the Stability of the Insulin Hexamer

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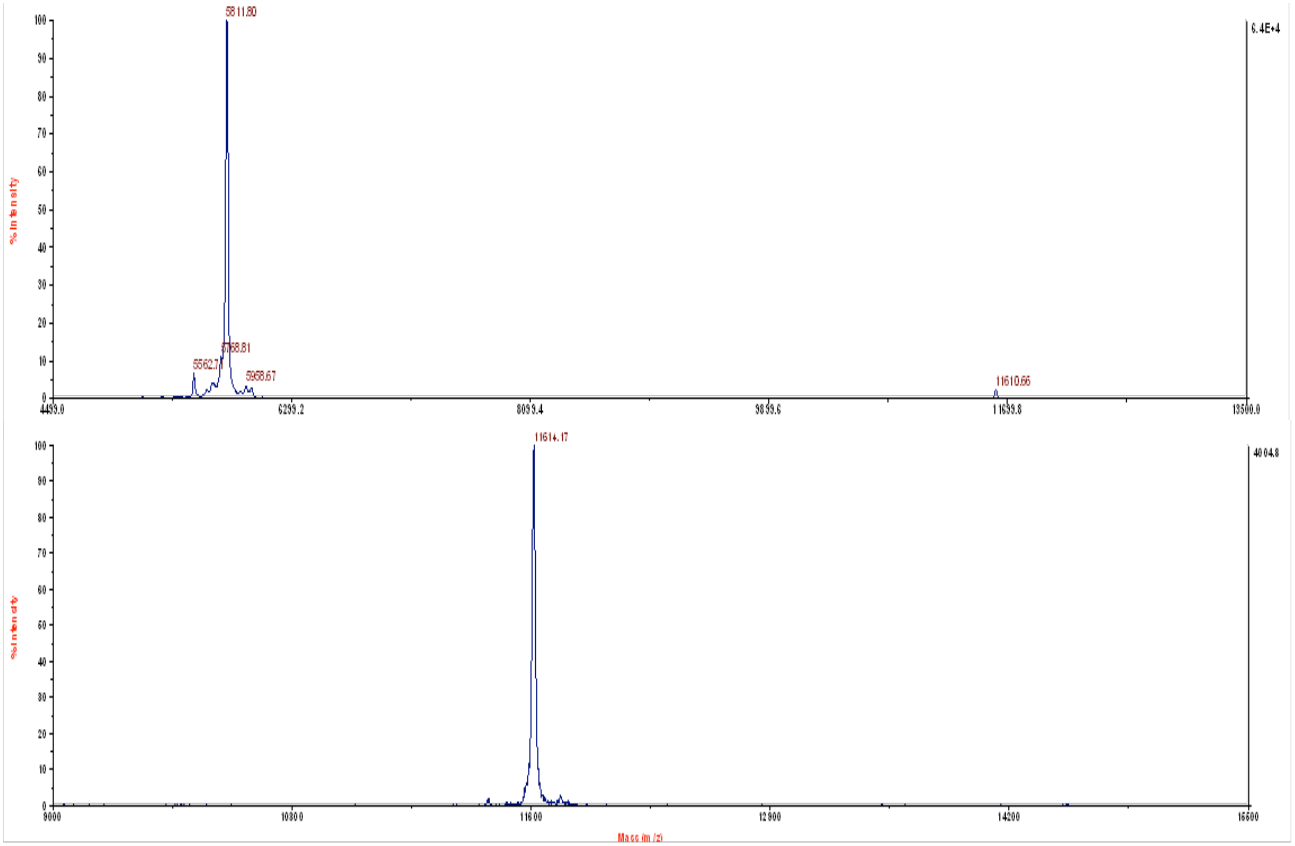

Figure S1. MALDI-TOF mass spectra of (**top**) 25 µM human insulin, showing predominantly monomer (*m/z* 5811.60), and (**bottom**) 75 µM human insulin, showing predominantly dimer (*m/z* 11614.17).

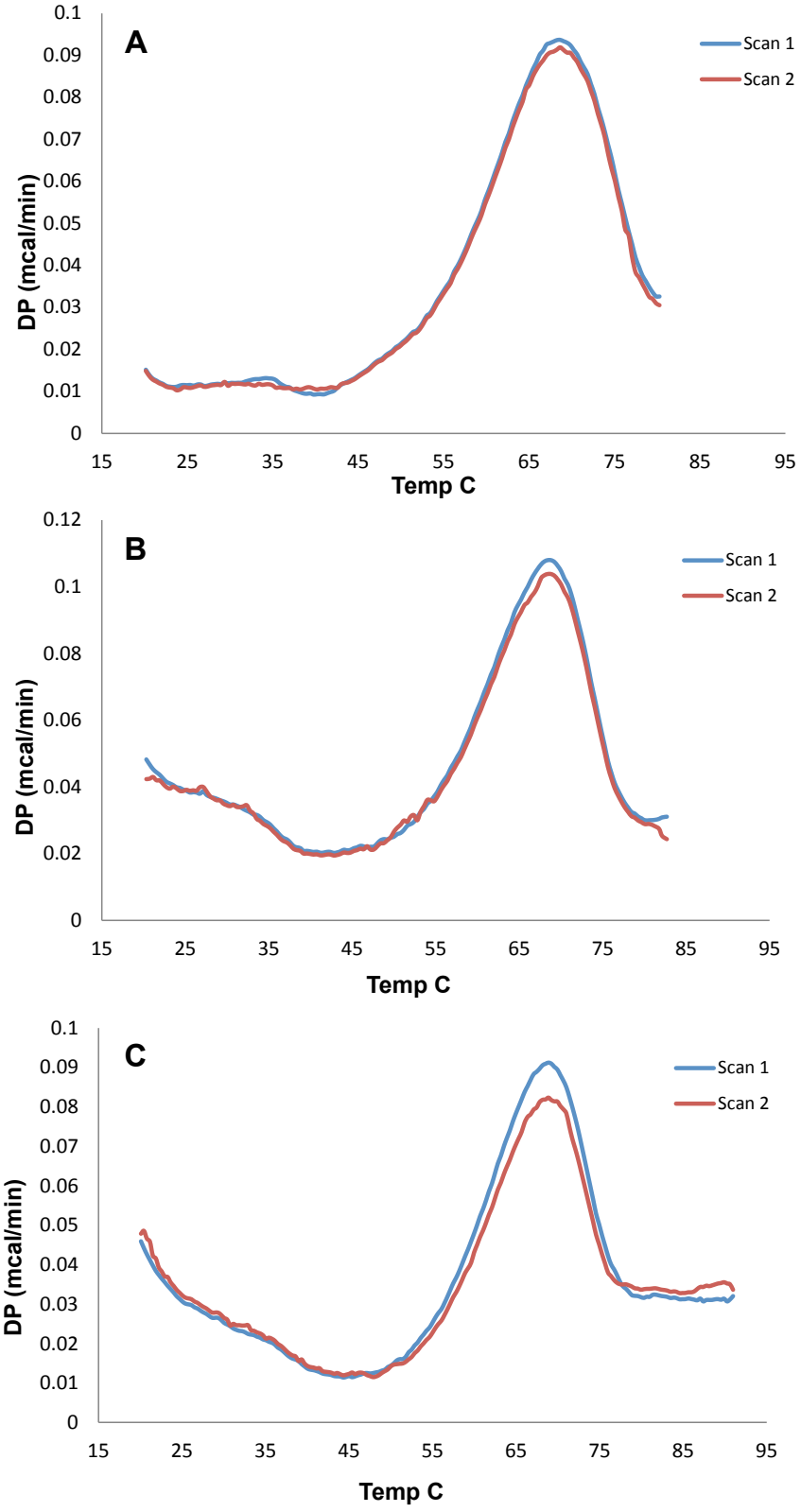

Figure S2. Representative DSC scans of T_6 human insulin showing the thermal reversibility upon repeated heating to 80-90 °C and subsequent cooling to 20 °C.

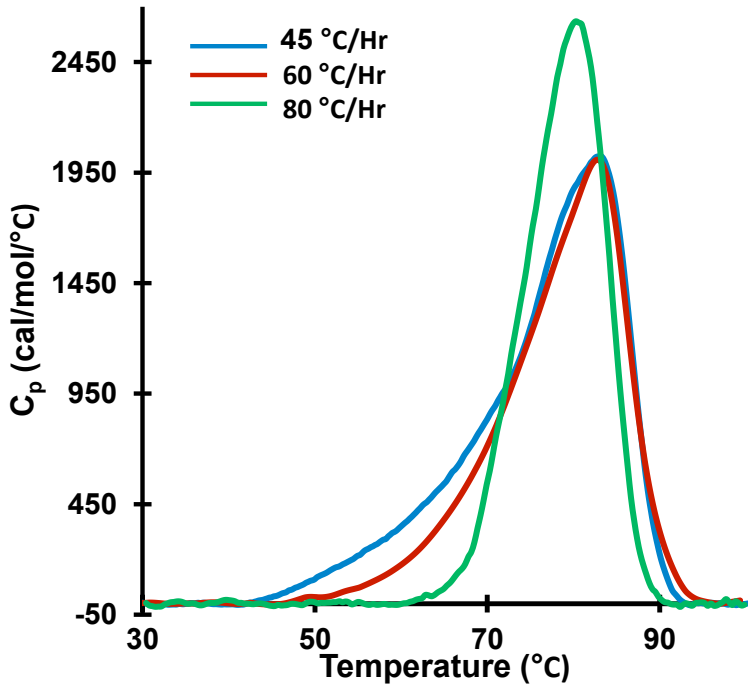

Figure S3. Representative baseline-adjusted, concentration-normalized DSC endotherms of 0.10 mM human insulin with the R_6' hexamer structure at scan rates of 45 °C/hr (blue), 60 °/hr (red) and 80 °C/hr (green).

Buffer	Parameters	Apo	T_6	T_6'	T_3R_3'
ACES	T_m (°C)	65 ± 1	77 ± 1	78 ± 1	79 ± 1
	ΔH_{cal} (kcal/mol)	12 ± 3	14 ± 1	16 ± 1	18 ± 1
Bis-Tris	T_m (°C)	74 ± 0.5	78 ± 0.2	83 ± 0.1	81
	ΔH_{cal} (kcal/mol)	18 ± 2	24 ± 4	30 ± 1	30
Tris	T_m (°C)	64 ± 0.3	78 ± 2	77	80 ± 1
	ΔH_{cal} (kcal/mol)	14 ± 1	15 ± 0.3	23	23 ± 1

Table S1. Average DSC parameters for 0.10 mM porcine insulin with the indicated hexamer structure in different buffers at 50 mM concentration, pH 7.4.

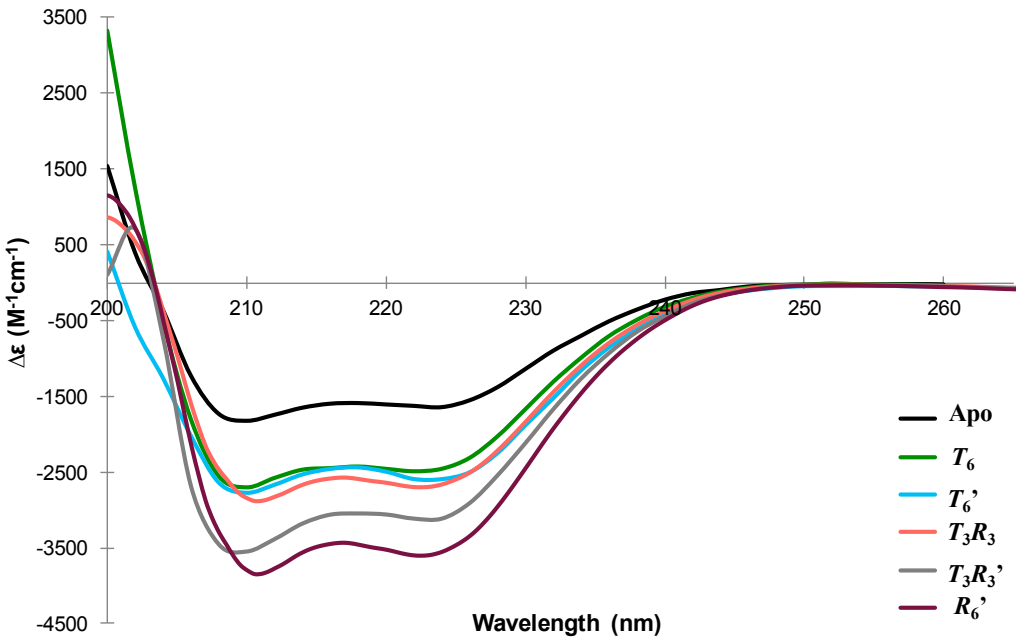

Figure S4. Room temperature UV-CD spectra of 40 μM human insulin with the indicated hexamer structure in 10 mM phosphate buffer, pH 7.4.

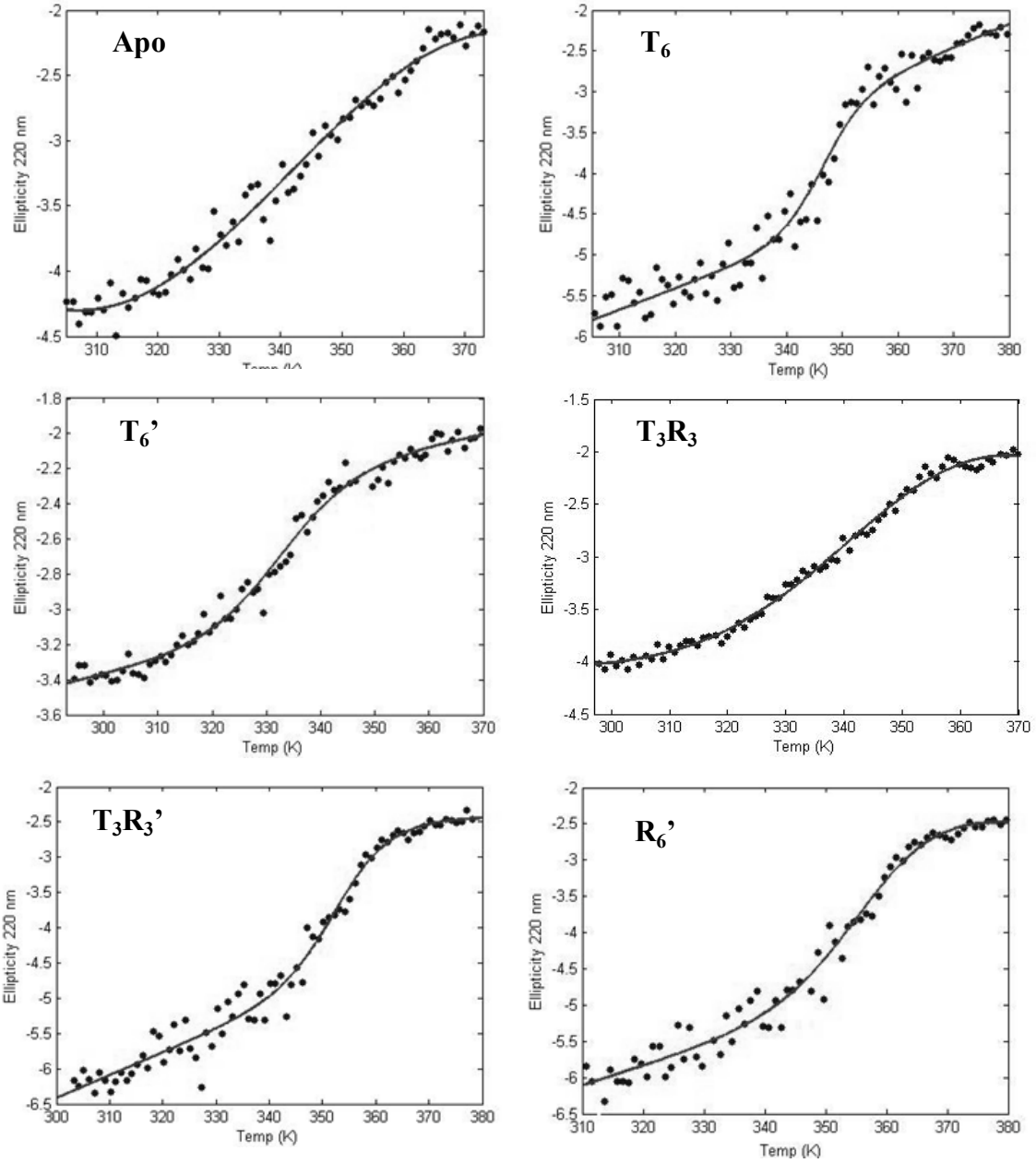

Figure S5. Representative plots of thermal unfolding monitored by CD at 220 nm for 10 μM Apo, T_6, T_6', T_3R_3, T_3R_3' and R_6' human insulin in 10 mM phosphate buffer, pH 7.4; best fit line from fitting the data to Equation 1.

Hexamer	ΔG_u (kcal/mol)[a]	ΔG_u (kcal/mol)[b]	ΔH_{cal} (kcal/mol)	ΔS_u (kcal/mol)
Apo	0	5.6 ± 0.6	64 ± 1	189 ± 3
T_6 (2 Zn^{2+}/Hex)	1.8 ± 1.0	11.1 ± 0.9	106 ± 2	311 ± 6
T_6 (9.6 Zn^{2+}/Hex)	10.1 ± 0.9	24.1 ± 1.2	174 ± 3	490 ± 7

Table S2. Thermodynamic values for the thermal unfolding of apo and of T_6 human insulin with stoichiometric and excess Zn^{2+} in 50 mM Tris buffer, pH 7.4; [a]338 K reference temperature; [b]298 K reference temperature.

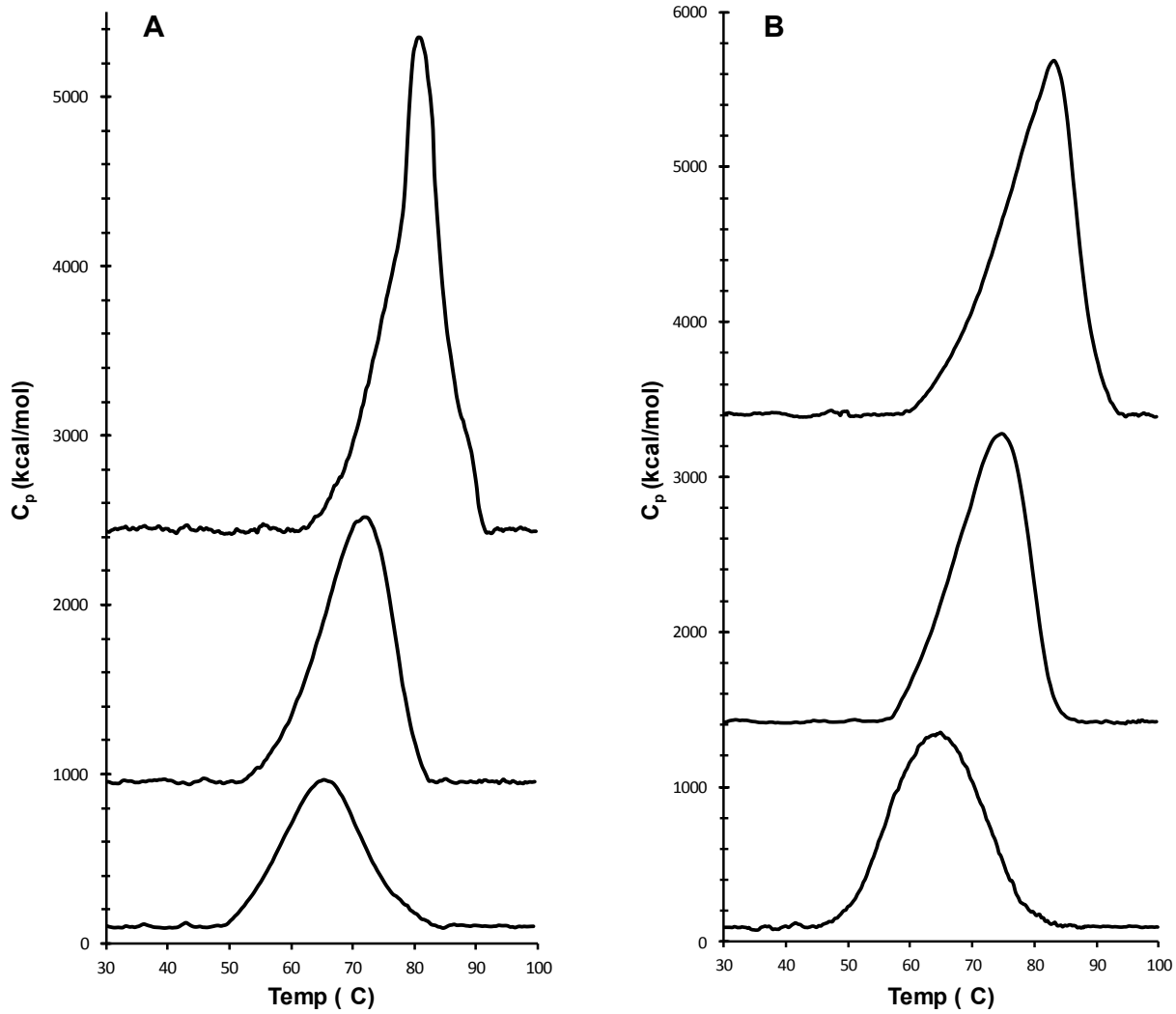

Figure S6. Representative baseline-adjusted, concentration-normalized DSC endotherms of 0.10 mM human insulin in **(A)** 50 mM Tris buffer, pH 7.4 and **(B)** 10 mM phosphate buffer, pH 7.4; **(bottom**) apo insulin, **(middle)** 2 Zn^{2+}/hexamer and **(top)** 9.6 Zn^{2+}/hexamer.

Materials and Methods

Chaotrope-induced protein unfolding involved addition of guanidine hydrochloride (GuHCl) to insulin samples in 10 mM sodium phosphate buffer, pH 7.4. Samples were incubated with GuHCl for two hours prior to ellipticity measurements. Changes in the protein 2° structure were monitored by the ellipticity at 220 nm measured with a JASCO J-815 CD spectrometer. The concentration of GuHCl at each titration point was determined by the refractive index.[1] Non-linear curve fitting was carried out using MATLAB and Equation 3, as described previously,[2]

$$f(x) = \frac{[(m_f x + b_f) + (m_u x + b_u)]\, exp\left[\frac{m(x - [D]_{50\%})}{RT}\right]}{1 + exp\left[\frac{m(x - [D]_{50\%})}{RT}\right]} \qquad (3)$$

where m_f, b_f and m_u, b_u are the slope and y-intercept for the folded (low [GuHCl]) and unfolded (high [GuHCl]) regions, R is the gas constant (8.314 J/mol K), T is the experimental temperature and $[D]_{50\%}$ is the concentration of denaturant at which 50% of the protein is unfolded (transition midpoint). The constant m has been discussed by Fersht and others,[2,3] and often provides an indication of the degree of unfolding. In this fitting model, x reflects changes with respect to the concentration of the denaturant.

1) Krivacic, J.R., and Urry, D.W. (1971) Ultraviolet refractive indices of aqueous solutions of urea and guanidine hydrochloride. *Analyt. Chem. 43*, 1508-1510.
2) Fersht A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*. W.H. Freeman & Company, Orange, VA.
3) Myers, J.K., Pace, C.N., and Scholtz, J.M. (1995) Denaturant *m* values and heat capacity changes: relation to changes in accessible surface area of protein unfolding. *Protein Sci. 4*, 2138-2148.

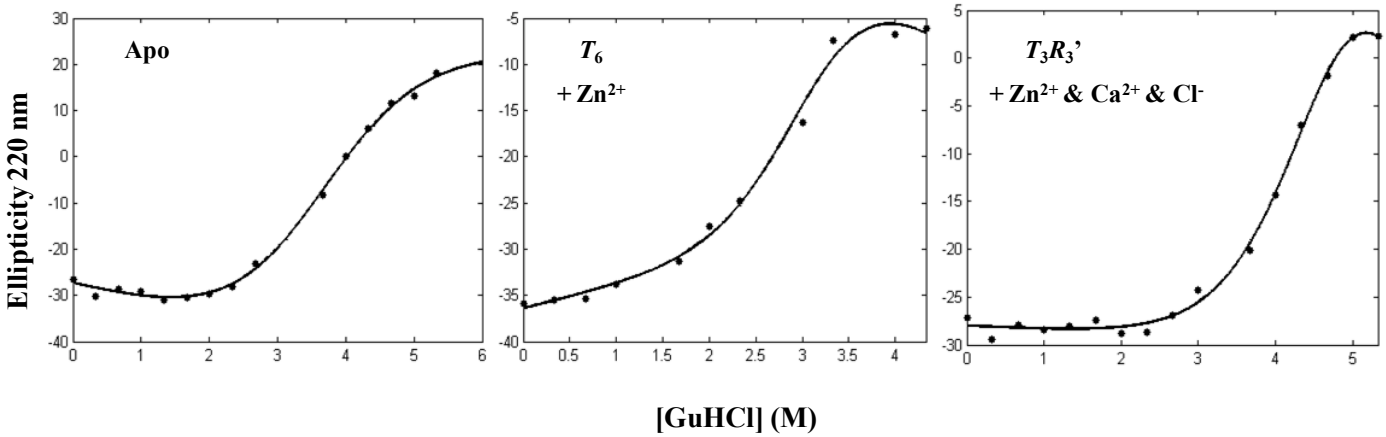

Figure S7. Representative plots of chaotrope (GuHCl) unfolding monitored by CD at 220 nm; best fit line from fitting the data to Equation 3, which gave the following *m* parameter and $[GuHCl]_{50\%}$, respectively: **Apo** (0.79 kcal/L, 3.6 M GuHCl), $\mathbf{T_6}$ (1.3 kcal/L, 3.1 M), $\mathbf{T_3R_3}$**'** (1.1 kcal/L, 4.6 M).

Hexamer	ΔG_u (kcal/mol)[a]	ΔG_u (kcal/mol)[b]	ΔH_{cal} (kcal/mol)	ΔS_u (cal/molK)[c]
Apo	0	5.6 ± 1.0	69 ± 3	211 ± 9
T_6	1.8 ± 2.0	7.9 ± 1.7	81 ± 3	238 ± 7
T_6'	3.9 ± 1.9	11.1 ± 1.8	100 ± 3	288 ± 8
T_3R_3	2.1 ± 1.1	9.5 ± 1.1	95 ± 3	279 ± 9
T_3R_3'	4.4 ± 2.7	10.7 ± 2.5	94 ± 2	269 ± 6
R_6'	9.5 ± 1.1	20.9 ± 1.3	153 ± 3	430 ± 8

Table S3. Thermodynamic values for the thermal unfolding of bovine insulin hexamers (2 Zn^{2+} per hexamer) determined by DSC on a per hexamer basis; [a]calculated with Equation 2 and 335 K (Apo T_m) reference temperature; [b]calculated with Equation 2 and 298 K reference temperature; [c]determined at T_m by $\Delta H_{cal}/T_m$.

Hexamer	ΔG_u (kcal/mol)[a]	ΔG_u (kcal/mol)[b]	ΔH_{cal} (kcal/mol)	ΔS_u (cal/molK)[c]
Apo	0	5.6 ± 1.0	69 ± 3	211 ± 9
T_6	9.0 ± 1.9	21.5 ± 1.9	158 ± 4	445 ± 10
T_6'	9.1 ± 1.1	21.7 ± 1.1	159 ± 3	448 ± 8
T_3R_3	6.5 ± 1.1	16.5 ± 1.2	130 ± 3	369 ± 9
T_3R_3'	6.1 ± 1.9	14.9 ± 1.8	119 ± 3	337 ± 8
R_6'	9.4 ± 1.8	21.4 ± 1.9	154 ± 4	431 ± 11

Table S4. Thermodynamic values for the thermal unfolding of bovine insulin hexamers (9.6 Zn^{2+} per hexamer) determined by DSC on a per hexamer basis; [a]calculated with Equation 2 and 335 K (Apo T_m) reference temperature; [b]calculated with Equation 2 and 298 K reference temperature; [c]determined at T_m by $\Delta H_{cal}/T_m$.