Supporting Information

"Thermodynamics of Formation of the Insulin Hexamer: Metal-Stabilized Proton-Coupled Assembly of Quaternary Structure"

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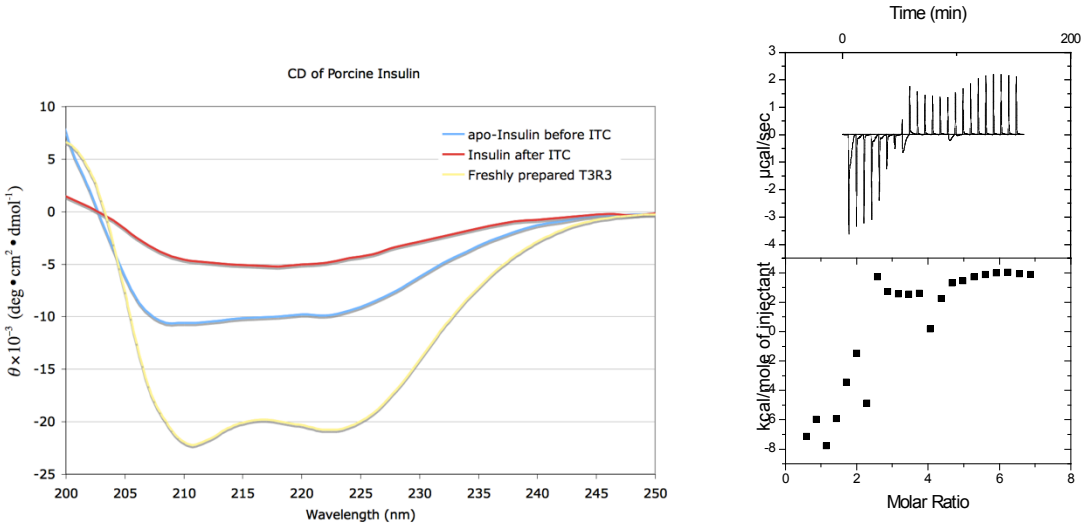

Figure S1. (left) UV CD spectra of insulin; blue spectrum: fresh insulin sample prior to ITC measurement; yellow spectrum: T_3R_3' sample prepared by direct addition of the appropriate stoichiometric amounts of Zn^{2+}, Ca^{2+} and Cl^- to insulin; red spectrum: T_3R_3' sample prepared by addition of Zn^{2+}, Ca^{2+} and Cl^- to insulin through an ITC experiment; (right) ITC thermogram of the insulin sample used for the red CD spectrum: 1.6 mM $ZnCl_2$ + 1.6 mM $CaCl_2$ → 0.05 mM porcine insulin monomer, 7 mM phosphate buffer, pH 7.4, 25 ± 0.2 °C.

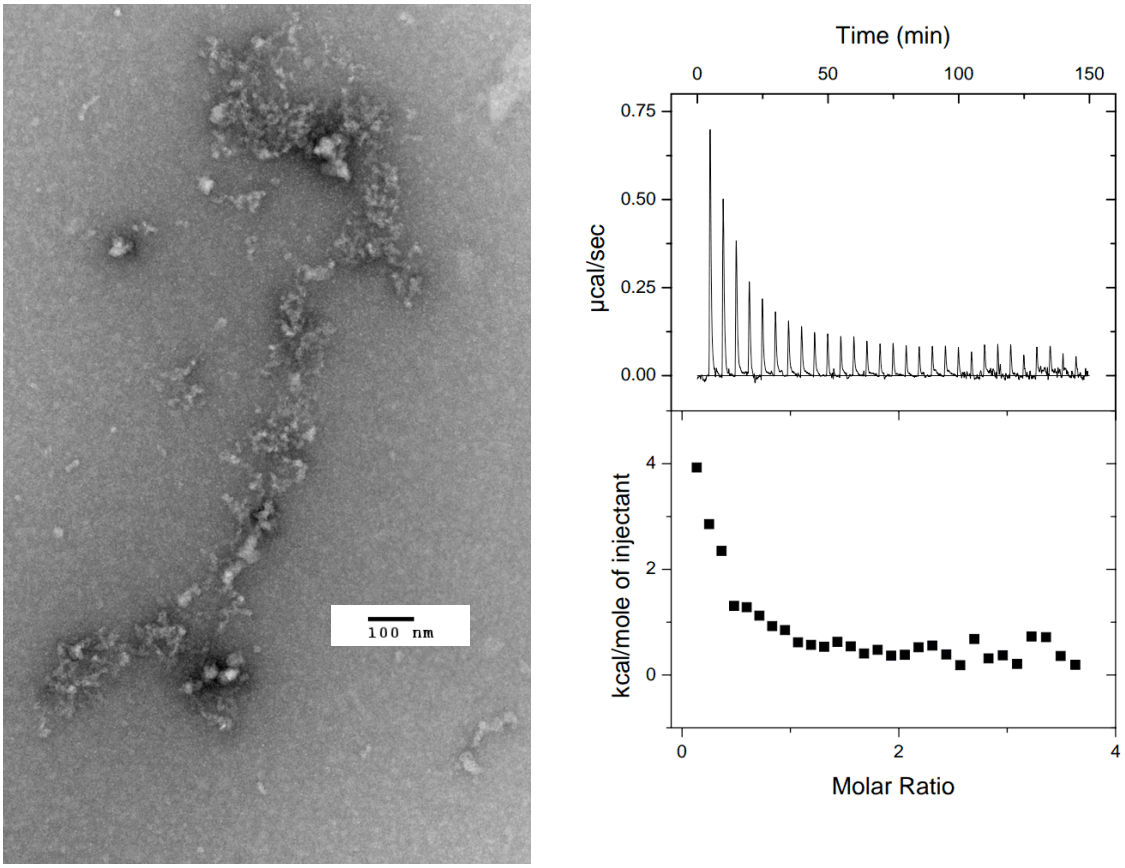

Figure S2. (left) Transmission electron microscopy (TEM) image of a T_3R_3' insulin sample prepared by addition of Zn^{2+}, Ca^{2+} and Cl^- to insulin through an ITC experiment; (right) ITC thermogram of T_3R_3' insulin sample used for the TEM image: 0.8 mM $ZnCl_2$ + 0.8 mM $CaCl_2$ → 0.05 mM porcine insulin monomer, 50 mM ACES buffer, pH 7.4, 25 ± 0.2 °C.

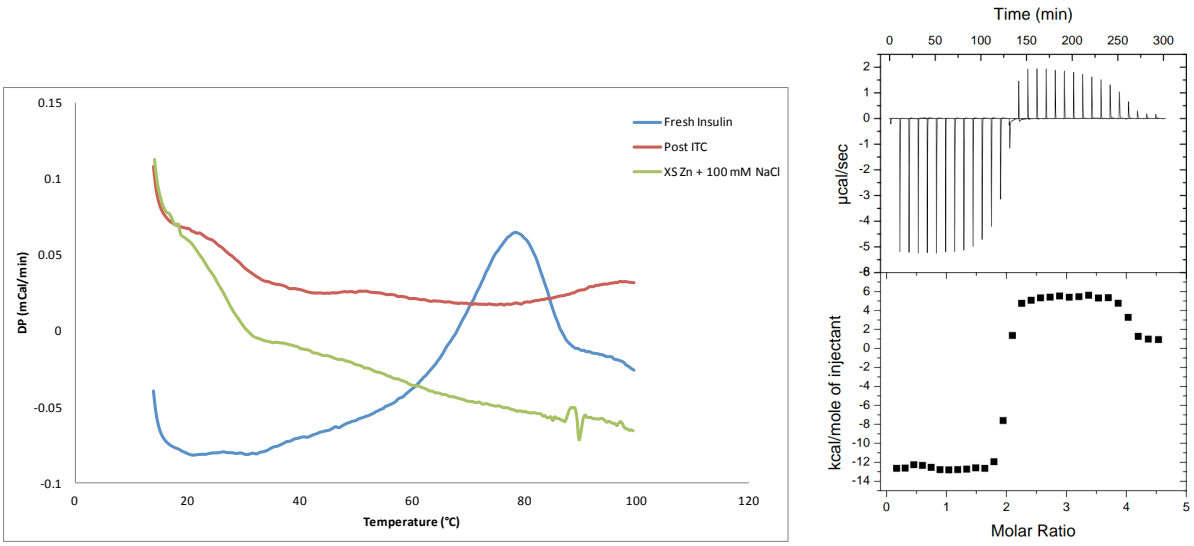

Figure S3. (left) DSC thermograms of insulin; blue: fresh T_3R_3' sample prepared by direct addition of the appropriate stoichiometric amounts of Zn^{2+}, Ca^{2+} and Cl^- to insulin; green: insulin sample with excess Zn^{2+} and NaCl added to concentrations known to induce aggregation (fibrillation); red: T_6' sample prepared by addition of Zn^{2+} and Ca^{2+} to insulin through an ITC experiment; (right) ITC thermogram of insulin sample used for the red DSC thermogram: 1 mM $ZnSO_4$ + 1 mM $CaSO_4$ → 0.05 mM porcine insulin monomer, 50 mM ACES buffer, pH 7.4, 25 ± 0.2 °C.

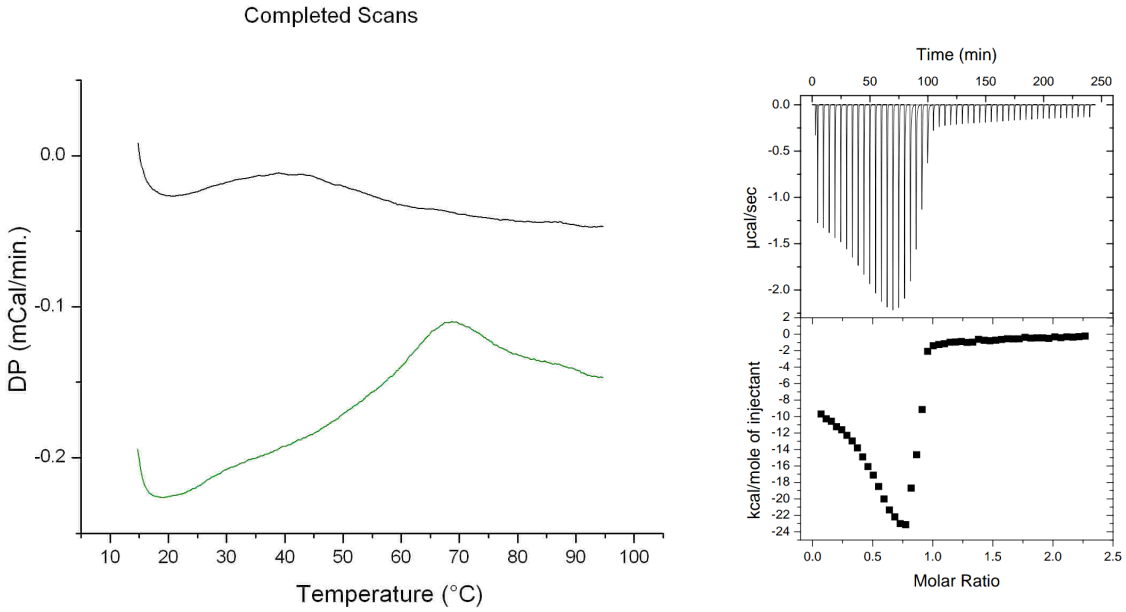

Figure S4. (left) DSC thermogram of insulin sample after ITC chelation measurement of T_3R_3 (green), with buffer baseline scan (black); (right) ITC thermogram of EDTA chelation of T_3R_3 insulin sample used for the DSC thermogram: 0.5 mM EDTA → 0.05 mM $ZnCl_2$ + 0.150 mM human insulin monomer, 50 mM Tris buffer, pH 7.4, 25 ± 0.2 °C.

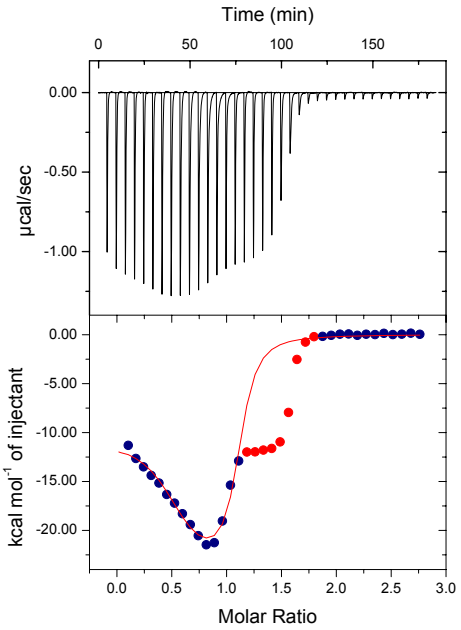

Figure S5. ITC thermogram of EDTA chelation of T_3R_3'; 0.28 mM EDTA → 0.027 mM $ZnCl_2$ + 0.013 mM $CaCl_2$ + 0.08 mM human insulin monomer in 50 mM Tris buffer, pH 7.4, 25 ± 0.2 °C; data attributed to Ca^{2+} chelation masked (red circles) for fit; best fit parameters using a two-sites binding model: n_1= 0.50 ± 0.01, K_1 = 5.0 (± 0.6) x 10^7, ΔH_1 = -10.6 ± 0.3 kcal·mol^{-1}; values for second site fixed at: n_2 = 0.58, K_2 = 4.3 x 10^6, ΔH_2 = -25 kcal·mol^{-1}.

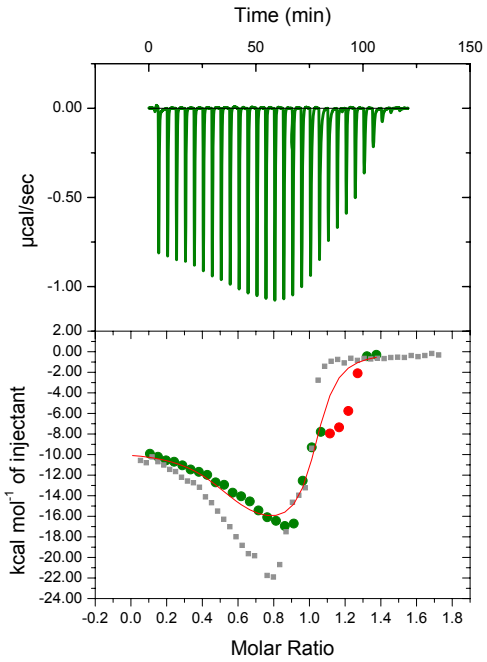

Figure S6. ITC thermogram of EDTA chelation of T_6; 0.28 mM EDTA → 0.027 mM $ZnSO_4$ + 0.08 mM human insulin monomer, 50 mM Tris buffer, pH 7.4, 25 ± 0.2 °C; data attributed to chelation of trace (<3 µM) Ca^{2+} masked (red circles) for fit; best fit parameters using a two-sites binding model: values for first site fixed at n_1= 0.513, K_1 = 7 x 10^7, ΔH_1 = -9.5 kcal·mol^{-1}; n_2 = 0.51 ± 0.01, K_2 = 5 (± 1) x 10^6, ΔH_2 = -18.6 ± 0.3 kcal·mol^{-1}.

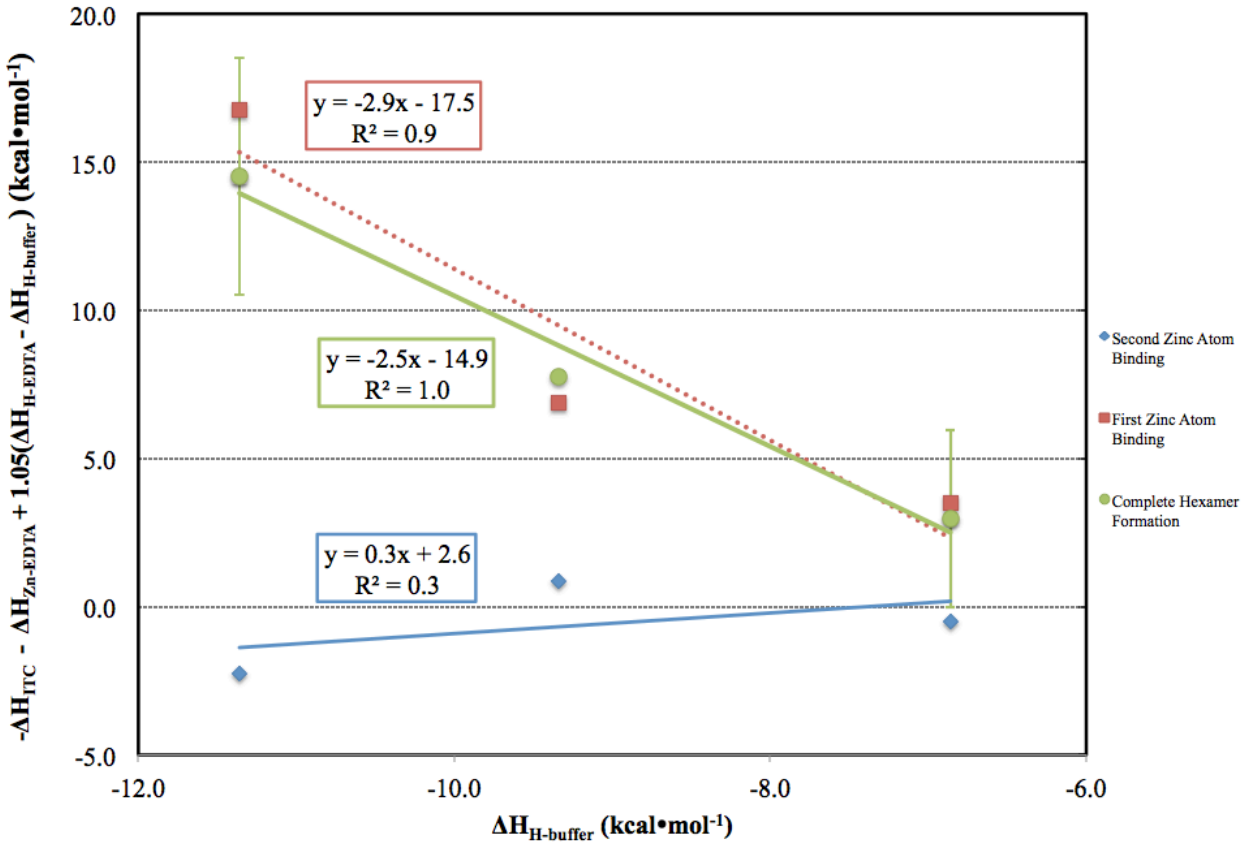

Figure S7. Plot of experimental ITC chelation enthalpies measured in Tris, Bis-Tris and ACES buffers to determine protonation/deprotonation contributions to the enthalpy at pH 7.4; red: first Zn^{2+} binding event; blue: second Zn^{2+} binding event; green: overall formation of the hexamer (sum of the two Zn^{2+} binding events). Plots are based on the sum of the contributions to the experimental enthalpy,

$$\Delta H_{ITC} = \Delta H_{EDTA-Zn} + 1.05\left(\Delta H_{Hbuffer} - \Delta H_{HEDTA}\right) + \Delta H_{Ins-Zn} + n(\Delta H_{Hbuffer})$$

which can be rearranged to give a linear relationship between the experimental enthalpy (ΔH_{ITC}) and the buffer protonation enthalpy ($\Delta H_{\mathrm{Hbuffer}}$).

$$\Delta H_{ITC} - \Delta H_{EDTA-Zn} + 1.05\left(\Delta H_{HEDTA} - \Delta H_{Hbuffer}\right) = \mathrm{n}\left(\Delta H_{Hbuffer}\right) + \Delta H_{Ins-Zn}$$

A plot of the experimental enthalpy, corrected for enthalpy contributions from Zn^{2+} binding to EDTA ($-\Delta H_{\mathrm{EDTA\text{-}Zn}} + 1.05(\Delta H_{\mathrm{HEDTA}} - \Delta H_{\mathrm{Hbuffer}})$), versus the buffer protonation enthalpy has a slope indicating the number of protons (n) binding to or released from insulin upon Zn^{2+} binding to the protein.

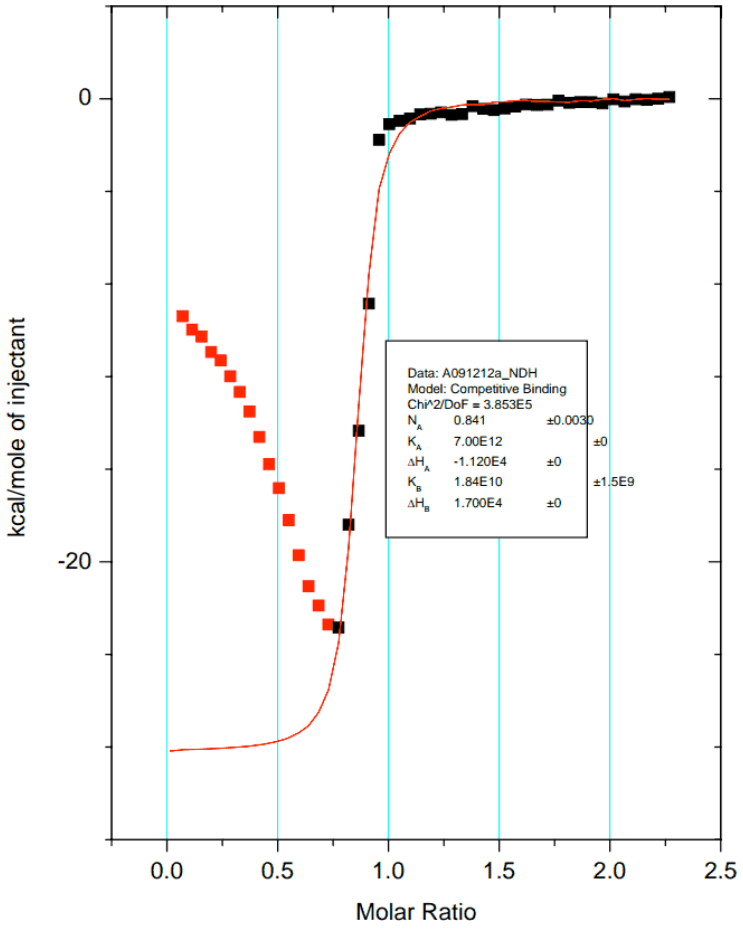

Figure S8. Best fit (red line) of the second event in the ITC thermogram of a representative EDTA chelation of T_3R_3 insulin using a competition binding model to determine the insulin-Zn^{2+} equilibrium constant; data due to the first event are masked (red squares) for the fit; input values for the fit are a) the stability constant of the EDTA-Zn^{2+} complex (7.0×10^{12}), determined from literature values and the experimental conditions, b) the enthalpy of formation of the EDTA-Zn^{2+} complex (-11.2 kcal/mol), determined independently for the experimental conditions, and c) the enthalpy of formation of the insulin-Zn^{2+} complex (17.0 kcal/mol), determined from the best fit of the entire thermogram to a two-sites binding model; experimental conditions are 50 mM Tris buffer, pH 7.4, 25 ± 0.2 °C.