van't Hoff and Calorimetric Enthalpies from Isothermal Titration Calorimetry: Are There Significant Discrepancies?†

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ABSTRACT: The enthalpy of a reaction is most often determined through one of two means; it can be determined directly using calorimetry or indirectly by measuring the temperature dependence of the equilibrium constant (i.e., the van't Hoff method). Recently, discrepancies have been noted between the enthalpy measured by calorimetry, ΔH°_{cal}, and the enthalpy determined by the van't Hoff method, ΔH°_{vH}. This has been suggested to indicate that the binding reaction is more complex than the simple one-to-one binding model used to describe the data. To better understand possible discrepancies between ΔH°_{cal} and ΔH°_{vH}, we have undertaken both experimental studies using isothermal titration calorimetry to measure the binding energetics of Ba^{2+} binding 18-crown-6 ether and 2′-CMP binding RNase A, along with a simulation of a system involving a molecule in conformational equilibrium coupled with binding. We find that when experimental setup and analysis are correctly performed, no statistically significant discrepancies between ΔH°_{cal} and ΔH°_{vH} exist even for the linked system.

The enthalpy change of a reaction, ΔH°, is a fundamental thermodynamic quantity that describes the amount of heat released or absorbed in the course of the reaction. For a reversible reaction it also describes the temperature dependence of the equilibrium constant, K, through the van't Hoff relationship,

$$\Delta H^\circ = -R\left(\frac{\partial \ln K}{\partial 1/T}\right) \quad (1)$$

where R is the gas constant, T is the absolute temperature, and the partial derivative emphasizes the fact that other experimental variables such as pressure are held constant.

The ΔH° of a reaction can, in general, be determined in one of two ways; it can be determined directly using calorimetry or indirectly by measuring the temperature dependence of the equilibrium constant. The latter is known as the van't Hoff method. In the van't Hoff method, the instantaneous slope of a plot of $\ln K$ vs $1/T$, multiplied by $-R$, is used to determine ΔH°. The instantaneous slope must be used because reactions in aqueous solutions are usually accompanied by a significant change in heat capacity, ΔC_p, which results in a temperature-dependent ΔH°. The errors induced by a small ΔC_p have been discussed previously by Chaires (*1*). Although both methods can be used to determine ΔH°, in the past few years the equivalence of ΔH° determined from both calorimetric and van't Hoff methods has been called into question (*2*−*6*)

The observed differences in the enthalpy determined calorimetrically, ΔH°_{cal},[1] and the enthalpy determined by the van't Hoff method, ΔH°_{vH}, have been interpreted as indicating that the studied reaction is more complicated than the simple one-to-one binding model used to describe the equilibrium. It has been noted that binding reactions can include the displacement of solvent and counterions, as well as other linked equilibria such as protonation or conformational changes. It has been suggested that ΔH°_{cal} includes these contributions but that they are not observed in ΔH°_{vH} (*7*). This suggests that the van't Hoff enthalpy is the "intrinsic" binding heat, whereas the calorimetric enthalpy includes other, concomitant reactions (i.e., linked equilibria).

If the ΔH°_{cal} and ΔH°_{vH} are, indeed, different, there is a fundamental deficiency in our understanding of solution thermodynamics, and it precludes the use of the van't Hoff analysis as a means of determining ΔH°, even for simple reactions. To gain a better understanding of possible discrepancies between ΔH°_{cal} and ΔH°_{vH}, we have measured the binding energetics of Ba^{2+} binding 18-crown-6 ether and 2′-CMP binding RNase A and have simulated a more complicated system. We find that when experimental setup and analysis are performed correctly there is no statistically significant difference between the ΔH° determined calorimetrically or by the van't Hoff method. We also find that even a complex model, such as the binding of a molecule to a protein in conformational equilibrium, is not expected to show differences between ΔH°_{cal} and ΔH°_{vH} even though it does add unusual temperature dependencies to the thermodynamics.

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[1] Abbreviations: ΔH°_{cal}: calorimetric enthalpy, ΔH°_{vH}: van't Hoff enthalpy, $\Delta C_{p,cal}$: calorimetric heat capacity, $\Delta C_{p,vH}$: van't Hoff heat capacity, ITC: isothermal titration calorimetry, 2′-CMP: 2′-cytidine monophosphate.

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MATERIALS AND METHODS

Materials. Barium chloride dihydrate (catalog no. B-6394), 18-crown-6 (catalog no. C-5515), RNase A (catalog no. R-5500), and 2′-CMP (catalog no. C-7137) were purchased from Sigma-Aldrich Company (St. Louis, MO).

Isothermal Titration Calorimetry. ITC experiments were performed from 5 to 45 °C using a Calorimetry Sciences Corporation model 4200 titration calorimeter (Calorimetry Sciences Corp., Spanish Fork, UT). The calorimeter was routinely calibrated with 500 μJ electrical pulses and by measuring the protonation heats of Tris buffer.

Barium chloride was dried overnight at 105 °C prior to weighing. Barium chloride and 18-crown-6 solutions were made at 0.2 and 0.02 M, respectively, in volumetric flasks and solutions were degassed before loading. The pH of the solutions was not adjusted, but no change in pH was noted after the reaction, which indicates an absence of any proton-linkage.

RNase A was dissolved in acetate buffer and then dialyzed for 16 h at 4 °C against 4 L of acetate buffer using 3500 MWCO dialysis tubing. Acetate buffer was prepared with 200 mM sodium acetate and 200 mM sodium chloride at pH 5.5. The dialysate was then used to make the 2′-CMP solution. The concentration of the RNase A solution was determined spectrophotometrically using an extinction coefficient of 9800 cm^{-1} M^{-1} at 280 nm (*8*). The concentration of the 2′-CMP solution was determined spectrophotometrically by diluting 1 mL of solution to 25 mL using 100 mM PBS (pH 7.0), then using an extinction coefficient of 7400 cm^{-1} M^{-1} at 260 nm (*8*).

ITC experiments were set up with 25 10-μL injections with one or two experiments performed at a given temperature. The integrated peaks from each experiment were used for data analysis with the exception of the initial injection. The first injection was routinely discarded for each experiment due to the possibility of diffusion across the needle or misalignment of the plunger. ITC data were analyzed using Bindworks 3.0 (Applied Thermodynamics, 1999)

van't Hoff Analysis. The van't Hoff analysis is defined by dividing the following relationship by T and taking the derivative with respect to $1/T$:

$$\Delta G^\circ = -RT\ln K = \Delta H^\circ_{\mathrm{Ref}} - T\Delta S_{\mathrm{Ref}}{}^\circ + \Delta C_p\left[(T - T_{\mathrm{Ref}}) - T\ln\left(\frac{T}{T_{\mathrm{Ref}}}\right)\right] \quad (2)$$

Therefore,

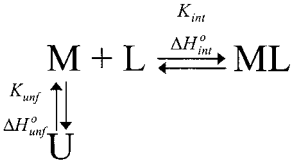
$$-R\left(\frac{\partial \ln K_{\mathrm{obs}}}{\partial \frac{1}{T}}\right) = \Delta H^\circ_{\mathrm{vH,Ref}} + \Delta C_{p,\mathrm{vH}}(T - T_{\mathrm{Ref}}) \quad (3)$$

where ΔH° and ΔC_p are now defined as the van't Hoff enthalpy and heat capacity change, $\Delta H^\circ_{\mathrm{vH,Ref}}$ and $\Delta C_{p,\mathrm{vH}}$, K_{obs} is the observed binding constant, R is the gas constant, T is the temperature of interest and T_{ref} is the reference temperature. The van't Hoff plots were fit to determine $\Delta H^\circ_{\mathrm{vH,Ref}}$ and $\Delta C_{p,\mathrm{vH}}$ with a reference temperature of 25 °C using KaleidaGraph v3.0 for Windows (Synergy Software).

Error Analysis. Errors are presented as ± one standard deviation. When necessary the error was propagated using the general form (*9*):

$$(df)^2 = \left[\left(\frac{\partial f}{\partial x}\right)^2_y (dx^2) + \left(\frac{\partial f}{\partial y}\right)^2_x (dy^2) + \cdots\right] \quad (4)$$

Scheme 1: Model Describing a Macromolecule Conformational Change Linked to Ligand Binding[a]



[a] M is the macromolecule, L is the ligand, and ML is the macromolecule−ligand complex. K_{int} and $\Delta H^\circ_{\mathrm{int}}$ are the intrinsic energetics for ligand−macromolecule binding. K_{unf} and $\Delta H^\circ_{\mathrm{unf}}$ are the energetics associated with macromolecule unfolding.

Binding Simulation. The calorimetric and van't Hoff enthalpies were simulated for a system where macromolecule folding and binding are linked (Scheme 1). The observed binding constant, K_{obs}, includes contributions from both the binding and unfolding equilibria. It is defined as follows:

$$K_{\mathrm{obs}} = \frac{K_{\mathrm{int}}}{1 + K_{\mathrm{unf}}} \quad (5)$$

where K_{int} is the intrinsic binding constant for binding the ligand to the folded macromolecule and K_{unf} is the unfolding constant for the macromolecule. The observed enthalpy is described by:

$$\Delta H^\circ_{\mathrm{obs}} = \Delta H^\circ_{\mathrm{int}} - \frac{K_{\mathrm{unf}}}{1 + K_{\mathrm{unf}}}\Delta H^\circ_{\mathrm{unf}} \quad (6)$$

where $\Delta H^\circ_{\mathrm{int}}$ is the intrinsic enthalpy for ligand-macromolecule binding, the ratio $K_{\mathrm{unf}}/(1+ K_{\mathrm{unf}})$ describes the fraction of unfolded macromolecule, and $\Delta H^\circ_{\mathrm{unf}}$ is the enthalpy of macromolecule unfolding. Parameters were chosen so that half of the macromolecule is in its folded, binding competent state at 25 °C. Simulation parameters were $K_{\mathrm{int}} = 4 \times 10^{10}$, $\Delta H^\circ_{\mathrm{int}} = -2.5$ kJ/mol, $\Delta S^\circ_{\mathrm{int}} = 195$ J mol^{-1} K^{-1}, $\Delta C_p = -1.1$ kJ mol^{-1} K^{-1}, $K_{\mathrm{unf}} = 1$, $\Delta H^\circ_{\mathrm{unf}} = 133$ kJ/mol, $\Delta S^\circ_{\mathrm{unf}} = 446$ J mol^{-1} K^{-1}, $\Delta C_{p,\mathrm{unf}} = 7.6$ kJ mol^{-1} K^{-1}, $T_{\mathrm{ref}} = 298$ K.

RESULTS AND DISCUSSION

To investigate potential discrepancies between the calorimetric enthalpy, $\Delta H^\circ_{\mathrm{cal}}$, and the van't Hoff enthalpy, $\Delta H^\circ_{\mathrm{vH}}$, two binding systems were examined, Ba^{2+} binding to the crown ether, 18-crown-6, and 2′-CMP binding to RNase A. Both systems are frequently used for ITC test reactions and calibration. Our values for the binding of Ba^{2+} to 18-crown-6 at 25 °C agree well with the values found by Briggner and Wadsö (*10*) ($K = 5900 \pm 200$ and $\Delta H^\circ_{\mathrm{cal}} = -31.4 \pm 0.2$ kJ/mol) but differ somewhat from the values published by Liu and Sturtevant (*5*) ($K = 2490 \pm 20$ and $\Delta H^\circ_{\mathrm{cal}} = -27.1$ kJ/mol).

Our data for the binding of 2′-CMP to RNase A cannot be compared to those of Naghibi and Sturtevant (*4*) because theirs were measured in 0.5 M sucrose. However, binding data for 2′-CMP binding to RNase A have been determined by other investigators (*8*, *11*). Comparison is difficult since

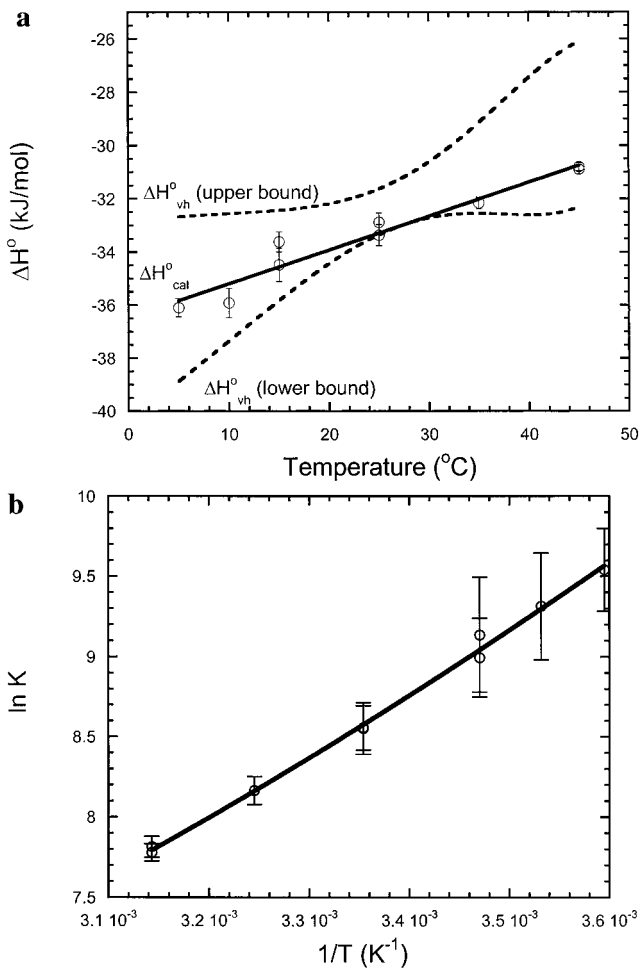


FIGURE 1: (A) The ΔH°_{cal} and upper and lower ΔH°_{vH} error bounds of Ba^{2+} binding 18-crown-6 as a function of temperature. (B) van't Hoff plot: ln*K* vs 1/*T* for Ba^{2+} binding 18-crown-6.

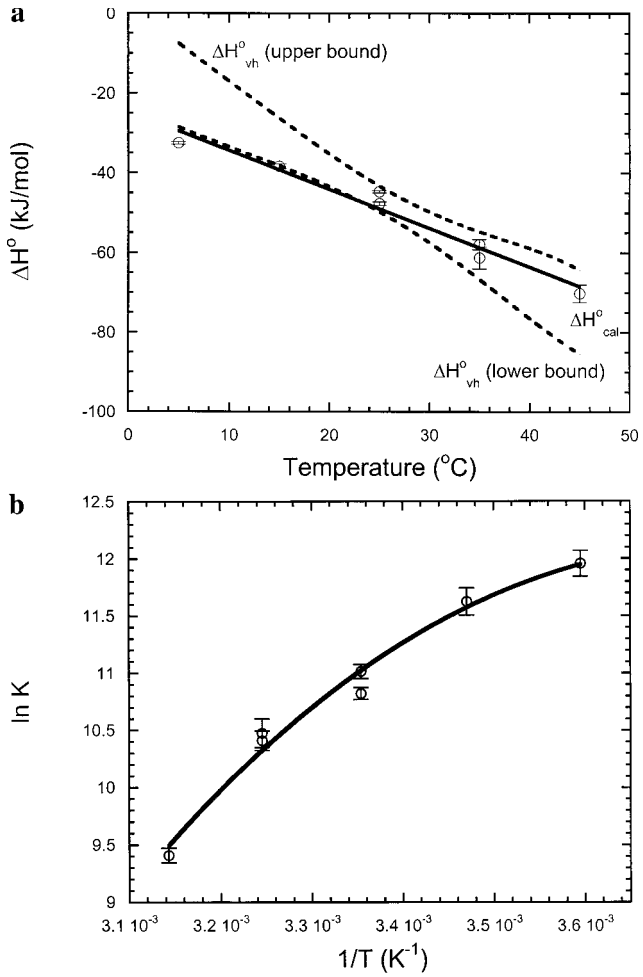


FIGURE 2: (A) The ΔH°_{cal} and upper and lower ΔH°_{vH} error bounds of 2′-CMP binding RNase A as a function of temperature. (B) van't Hoff plot: ln*K* vs 1/*T* for 2′-CMP binding RNase A.

Table 1: ΔH°_{cal} and Equilibrium Constant (*K*) Values for Ba^{2+}/18-crown-6 and 2-CMP/RNase A Binding

temp	$BaCl_2$/18-Crown−6		2′CMP/RNase	
(°C)	ΔH°_{cal} (kJ/mol)	K/10^3	ΔH°_{cal} (kJ/mol)	*K*/10^4
5	−36.1 ± 0.4	14 ± 3.6	−32.6 ± 0.4	15.6 ± 1.7
10	−35.9 ± 0.6	11 ± 3.7		
15	−33.6 ± 0.4	8.1 ± 2.0	−38.3 ± 0.6	11.2 ± 1.3
	−34.5 ± 0.6	9.3 ± 3.3		
25	−33.4 ± 0.4	5.2 ± 0.8	−44.7 ± 0.3	6.1 ± 0.4
	−32.9 ± 0.4	5.0 ± 0.7	−47.7 ± 0.5	5.0 ± 0.3
35	−32.2 ± 0.2	3.5 ± 0.3	−57.9 ± 1.3	3.3 ± 0.3
			−61.3 ± 2.7	3.5 ± 0.4
45	−30.9 ± 0.2	2.5 ± 0.2	−70.2 ± 2.2	1.20 ± 0.08
	−30.8 ± 0.2	2.4 ± 0.1		

Table 2: Comparisons between Calorimetric and van't Hoff Enthalpies and Heat Capacity Changes at 25 °C

	calorimetric	van't Hoff
	$BaCl_2$/18-Crown−6	
ΔH° (kJ/mol)	−33.3 ± 0.2	−32.5 ± 0.9
ΔC_p (J/mol/K)	127 ± 10	160 ± 150
	2′-CMP/RNase	
ΔH° (kJ/mol)	−49 ± 1	−46 ± 3
ΔC_p (J/mol/K)	−970 ± 90	−1400 ± 500

the interaction is highly dependent on such variables as protein and salt concentration as well as pH. It is important to note that we are concerned here with comparing ΔH°_{cal} and ΔH°_{vH} obtained from the same experiments.

The binding constant, *K*, and ΔH°_{cal} were determined as a function of temperature using isothermal titration calorimetry (ITC) (see Table 1). Evaluation of the binding constant as a function of temperature yielded ΔH°_{vH}. Figure 1, panel A shows the temperature dependence of ΔH°_{cal} and the propagated error range of ΔH°_{vH} for Ba^{2+} binding 18-crown-6, while Figure 1, panel B is the corresponding van't Hoff plot. From 5 to 45 °C, Ba^{2+}/18-crown-6 binding possesses exothermic ΔH°_{cal} values and a small positive $\Delta C_{p,cal}$. Figure 2, panel A shows the temperature dependence of ΔH°_{cal} and the propagated error range of ΔH°_{vH} for 2′-CMP/RNase A binding, while Figure 2, panel B is the corresponding van't Hoff plot. The 2′-CMP/RNase A binding also displays negative ΔH°_{cal} values but possesses a negative $\Delta C_{p,cal}$.

Calorimetric and van't Hoff ΔH° and ΔC_p values for both Ba^{2+}/18-crown-6 and 2′-CMP/RNase A are shown in Table 2. For both binding systems, ΔH°_{cal} and ΔH°_{vH} values are in excellent agreement. The calorimetric heat capacity change, $\Delta C_{p,cal}$, and the van't Hoff heat capacity change, $\Delta C_{p,vH}$, are also in agreement. It is worth noting that the error associated with $\Delta C_{p,vH}$ using the van't Hoff method is considerably larger than that found calorimetrically, $\Delta C_{p,cal}$. This is a consequence of $\Delta C_{p,vH}$ coming from the second derivative of the van't Hoff plot, whereas $\Delta C_{p,cal}$ is determined by the slope (i.e., the first derivative) of ΔH°_{cal} vs *T*.

Table 3: $\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$ Ratios for Ba^{2+}/18-crown-6 and 2′-CMP/RNase A Binding Extrapolated to Various Temperature[a]

temp (°C)	ΔH°_{cal}	ΔH°_{vH}	$\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$
	$BaCl_2$/18-Crown-6		
5	-35.8 ± 0.3	-35.8 ± 3.1	1.00 ± 0.09
15	-34.6 ± 0.2	-34.1 ± 1.7	1.01 ± 0.05
25	-33.3 ± 0.2	-32.5 ± 0.9	1.02 ± 0.03
35	-32.0 ± 0.2	-30.8 ± 1.7	1.04 ± 0.06
45	-30.7 ± 0.3	-29.2 ± 3.1	1.05 ± 0.11
	2′-CMP/RNase A		
5	-29.5 ± 2.0	-18.0 ± 10.5	1.64 ± 0.97
15	-39.2 ± 1.4	-32.2 ± 6.0	1.22 ± 0.23
25	-49.0 ± 1.1	-46.5 ± 3.2	1.05 ± 0.08
35	-58.7 ± 1.4	-60.7 ± 6.0	0.97 ± 0.10
45	-68.5 ± 2.0	-74.9 ± 10.5	0.91 ± 0.13

[a] The reference temperature is 25 °C. Enthalpies were extrapolated using $\Delta H^\circ_{(T)} = \Delta H^\circ_{(25°C)} + \Delta C_p(T - T_R)$. [b] Enthalpy units are kJ/mol.

To further compare ΔH°_{cal} and ΔH°_{vH}, the ratio of these values are examined over the experimental temperature range (Table 3). If there is no discrepancy, that is if calorimetric and van't Hoff enthalpies are the same, the $\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$ ratio will equal one within error. The enthalpy value at each temperature, $\Delta H°(T)$, is determined using eq 3 through extrapolation using ΔH°_{ref} and ΔC_p values determined at the reference temperature of 25 °C. Errors associated with ΔH°_{ref} and ΔC_p dictate the errors associated with the extrapolated enthalpy values. It is necessary to propagate these errors to determine whether the $\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$ ratio is significantly different than one. For both Ba^{2+}/18-crown-6 and 2′-CMP/RNase the $\Delta H^\circ_{cal}/\Delta H^\circ_{vH}$ ratio was one within error throughout the studied temperature range. Although a ratio of 1.64 (2′-CMP/RNase at 5 °C) may appear significant, the associated error is quite large ($\pm$ 0.97). The error increases for temperatures more distant from the reference temperature since the error associated with $\Delta C_{p,vH}$ is large, especially when ΔC_p is small (*1*).

While recent investigations have questioned the equivalence between ΔH°_{cal} and ΔH°_{vH}, we observe no significant discrepancies for these relatively simple binding reactions. It has been suggested that the observed discrepancies between ΔH°_{cal} and ΔH°_{vH} could be due to binding reactions that are more complicated than is suggested by the one-to-one binding scheme used to model the data. Additional equilibria, such as displacement of solvent, counterions, or protons, as well as conformational equilibria, could be contributing differently to the calorimetric and van't Hoff enthalpies.

To determine whether additional equilibria could produce such discrepancies, a simulation was analyzed in which a macromolecule−ligand interaction occurs where the macromolecule is in equilibrium between a native, binding competent form, and an unfolded, binding incompetent form (see Scheme 1). The simulation is set up so that the transition temperature between folded and unfolded macromolecule is within the experimental temperature range. The simulated temperature dependence of the enthalpy and van't Hoff plot is shown in Figure 3, panels A and B. The presence of the additional folding equilibrium, results in unusual temperature dependence of the binding constant and the $\Delta H°$. However, this unusual temperature dependence is manifested in both the calorimetric and the van't Hoff enthalpies so that they are equal over the entire temperature range (Figure 3). Consequently, the presence of an additional, linked equilibrium does not result in discrepancies between van't Hoff and calorimetric enthalpies even when the binding is incorrectly assumed to be a simple one-to-one reaction between native protein and ligand.

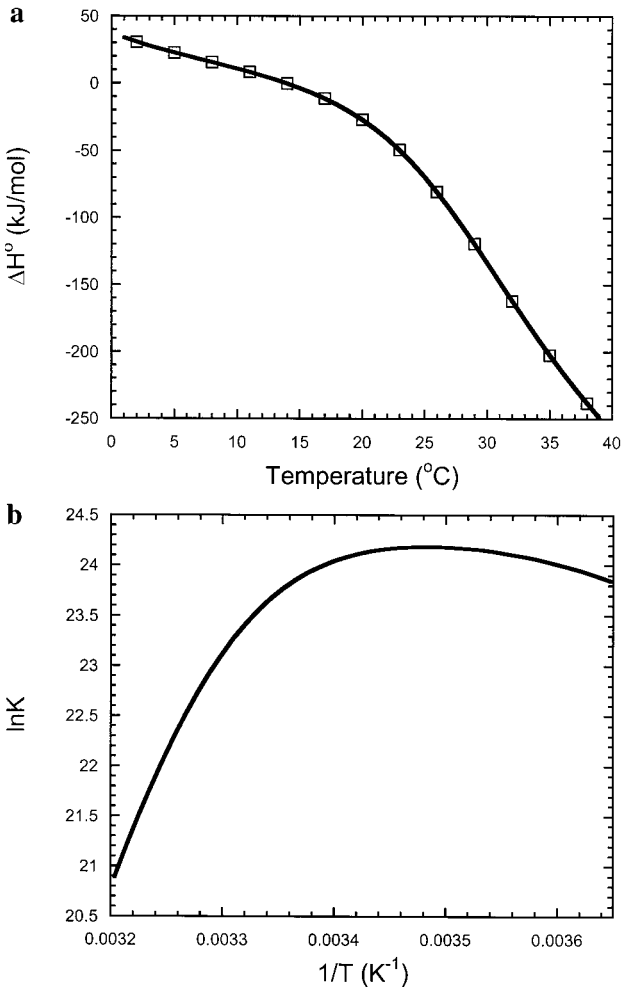


FIGURE 3: (A) Simulation of the observed calorimetric and van't Hoff enthalpy of binding for a macromolecule-ligand interaction with a linked unfolding equilibrium of the macromolecule. (□) calorimetric enthalpy, (—) van't Hoff enthalpy. (B) Simulation of the van't Hoff plot for the macromolecule−ligand interaction with a linked macromolecule unfolding equilibrium.

The most common type of linked equilibrium is proton linkage in which the binding reaction is pH dependent. Proton linkage results in additional complexities not observed in the conformational equilibrium case simulated here. While it is possible to design an experiment in which there are significant differences between ΔH°_{cal} and ΔH°_{vH} due to proton linkage, this represents an unusual case (Horn, Brandts, and Murphy, manuscript in preparation).

CONCLUSIONS

A combination of experimental and simulation studies performed here do not indicate that there are, or should be, significant discrepancies between binding enthalpies determined calorimetrically or using the van't Hoff method. Our analyses indicate that where such discrepancies are observed, they are likely to arise from either uncertainties due to extrapolation or inadequate calibration of the instrument. These differences (as in the former case) are not statistically

significant and are not indicative of linked equilibria such as conformational changes or changes in hydration.

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