van't Hoff and Calorimetric Enthalpies II: Effects of Linked Equilibria†

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ABSTRACT: The complexity of binding reactions, including the linkage with other equilibria, is becoming increasingly apparent in biological processes such as signal transduction. Understanding these interactions requires obtaining thermodynamic profiles for each of the equilibria that occur in a binding event. Concern has been raised as to whether linked equilibria contribute differently to thermodynamics, such as $\Delta H°$ and ΔC_p, obtained from calorimetric and van't Hoff methods. We have previously shown that linked equilibria do not contribute differently to the van't Hoff and calorimetrically determined $\Delta H°$ for processes such as linked folding or hydration. Here, examples of proton and ion linkage are examined. We show that there is no reason to expect the calorimetric and van't Hoff $\Delta H°$ to be different, even without prior knowledge of the presence or absence of linked equilibria, as long as the system is permitted to equilibrate. However, it is possible to create experimental scenarios that result in $\Delta H°_{cal}$ and $\Delta H°_{vH}$ discrepancies. Furthermore, it is found that the presence of linked equilibria in all cases can result in "nonconventional" $\Delta H°$ and ΔC_p profiles, making data analysis nontrivial.

When seeking to understand the thermodynamics of a biological binding reaction, one generally obtains the enthalpy, $\Delta H°$, which describes the amount of heat released or absorbed in the course of a reaction. The enthalpy is related to the nature of noncovalent forces (e.g., hydrogen bonds and van der Waals interactions) that are formed/lost between macromolecule(s) and/or solvent (*1*). Typically, $\Delta H°$ is determined in one of two ways: it may be determined directly by using calorimetry, $\Delta H°_{cal}$;[1] or it may be determined indirectly by measuring the temperature dependence of the equilibrium constant, known as the van't Hoff enthalpy, $\Delta H°_{vH}$.

Recently, the equivalence of these two methods has been questioned (*2*−*6*). We have shown, using both experiment and simulations, that when experimental setup and data analysis are correctly performed there is no deviation between $\Delta H°_{cal}$ and $\Delta H°_{vH}$. This was demonstrated for both simple binding and cases in which there is a conformational equilibrium linked to binding (*7*). Here, we examine the case of heterotropic linkage in which the binding of a second ligand is linked to the binding of the primary ligand.

Linked equilibria, although not always accounted for, are extremely common in biological binding events. Equilibria such as proton binding (*8*−*10*), ion binding (*11*), or conformational change (*12*−*14*) can be linked to a binding equilibria and will contribute to the overall binding energetics ($\Delta G°$, $\Delta H°$, $\Delta S°$, and ΔC_p). Therefore, when studying such binding, one must often distinguish the "intrinsic" thermodynamics that result from the binding interaction of interest (e.g., protein A binding protein B) from the "observed" or "apparent" thermodynamics, which includes contributions from both the binding equilibrium of interest and from the linked equilibria. Although $\Delta H°_{cal}$ includes contributions from linked equilibria, it has been suggested that $\Delta H°_{vH}$ will not (*15*), but rather will represent the "intrinsic" binding enthalpy (i.e., those contributions coming solely from the binding of the ligand and macromolecule).

Most biological thermodynamic studies aim to determine the energetics of a specific binding equilibrium. In principle, with knowledge of both the overall observed binding thermodynamics and those of the linked equilibria, one can calculate the thermodynamics of the binding reaction of interest (*16*). This would also allow the observed thermodynamics of binding to be calculated at any concentration of linked species. Alternatively, the experiment could be set up such that a single equilibrium is monitored in the course of the experiment. In practice, however, it is not generally known what or how many equilibria might be contributing to the observed thermodynamics and it is often impossible to isolate a specific equilibrium in a single experiment. Thus, in many cases, these additional contributions are ignored. With the likely occurrence of these complexities in many biological binding systems it is necessary to determine how linked equilibria affect $\Delta H°_{cal}$ and $\Delta H°_{vH}$ under various experimental conditions.

Here we address how complex equilibrium systems involving changes in proton or ion binding contribute to $\Delta H°_{cal}$ and $\Delta H°_{vH}$. This requires simulating the thermody-

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[1] Abbreviations: $\Delta H°_{cal}$, calorimetric enthalpy; $\Delta H°_{vH}$, van't Hoff enthalpy; $\Delta C_{p,cal}$, calorimetric heat capacity; $\Delta C_{p,vH}$, van't Hoff heat capacity; ITC, isothermal titration calorimetry.

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namic parameters for all equilibria involved as a function of temperature but analyzing the systems as though it were a simple, one-to-one binding. As with simple binding and linkage to conformational changes, we find that ΔH°_{cal} and ΔH°_{vH} are equivalent; however, this is true only when the system is allowed to equilibrate with temperature when measuring ΔH°_{vH}. In proton linked systems, the equivalence between the observed ΔH°_{cal} and ΔH°_{vH} (and similarly their temperature dependence $\Delta C_{p,cal}$ and $\Delta C_{p,vH}$) can deviate under experimental conditions where the proton concentration (i.e., the pH) is held constant over the temperature range studied. We describe the contributions to ΔH°_{cal} and ΔH°_{vH} in each of these common scenarios.

It should be noted that the aim of this paper is not to address how the intrinsic energetics can be obtained experimentally. Approaches have been described in the literature (*16*, *17*). Rather, the aim of this paper is to aid in the interpretation of experimental determinations of ΔH°, particularly when the systems have not been thoroughly characterized.

THEORY

van't Hoff Analysis. The van't Hoff enthalpy and heat capacity changes are determined by the temperature dependence of the binding constant. This can be shown by taking the derivative of the following relationship with respect to $1/T$:

$$\Delta G^\circ = -RT\ln K = \Delta H^\circ_{Ref} - T\Delta S^\circ_{Ref} + \Delta C_p\left((T - T_{Ref}) - T\ln\left(\frac{T}{T_{Ref}}\right)\right) \quad (1)$$

Therefore,

$$-R\left(\frac{\partial \ln K_{obs}}{\partial \frac{1}{T}}\right) = \Delta H^\circ_{vH,Ref} + \Delta C_{p,vH}(T - T_{Ref}) \quad (2)$$

where ΔH°_{Ref} and ΔC_p are now defined as the van't Hoff enthalpy and heat capacity change, $\Delta H^\circ_{vH,Ref}$ and $\Delta C_{p,vH}$, respectively, K_{obs} is the observed binding constant, R is the gas constant, T is the temperature of interest, T_{ref} is the reference temperature, and the partial derivative emphasizes that other experimental variables such as pressure are constant. The van't Hoff equation in this form should be used in cases where ΔC_p is nonzero.

The van't Hoff enthalpy is simulated by taking the numerical derivative of ln K_{obs} as a function of $1/T$ multiplied by $-R$. The binding constant K_{obs} is the observed binding constant which includes contributions from linked equilibria (discussed below). Each simulation is analyzed without accounting for linked equilibria, which represents a scenario in which one does not have prior knowledge of the additional equilibria occurring within the experiments.

Binding Simulation. The simulated system entailed a macromolecule−ligand interaction in which a proton can bind the free macromolecule or the macromolecule−ligand complex (see Scheme 1a). The observed binding constant, K_{obs}, includes both protonated and unprotonated macromolecule:

$$K_{obs} = \frac{[ML] + [M^+L]}{([M] + [M^+])[L]} = \frac{[ML]}{[M][L]}\cdot\frac{(1 + K_p^c[H^+])}{(1 + K_p^f[H^+])} = K_{int}\frac{(1 + K_p^c[H^+])}{(1 + K_p^f[H^+])} \quad (3)$$

where [ML] and [ML$^+$] are the unprotonated and protonated macromolecule−ligand complex, respectively, [M] is the free macromolecule, [L] is the free ligand, K_p^f and K_p^c are the proton binding constants for the free and complexed macromolecule, respectively, K_{int} is the intrinsic binding constant (i.e., in the absence of protonation), and [H$^+$] is the proton concentration (where [H$^+$] is taken as equivalent to the proton activity, a_{H^+}).

The contribution of proton linkage to the ΔH° of binding has been rigorously described by Baker and Murphy (*17*), and can be expressed as follows:

$$\Delta H^\circ(T) = \Delta H^\circ_{int}(T) - \bar{H}^f\Delta H_p^f + \bar{H}^c\Delta H_p^c + N_{H^+}\Delta H_i^b(T) \quad (4)$$

where ΔH°_{int} is the intrinsic enthalpy for ligand−macromolecule binding, $\bar{H}^f$ and $\bar{H}^c$ are the average number of protons bound to the free and complexed macromolecule, N_{H^+} is the number of protons released by the buffer upon binding, ΔH_p^f and ΔH_p^c are the enthalpies of protonation for the free and complexed macromolecule, and ΔH_i^b is the enthalpy of ionization of the buffer. The ΔC_p can be expressed analytically (*17*) or evaluated as a numerical derivative of ΔH°_{obs}. Simulation parameters are shown in Table 1. Note that neither the ligand-macromolecule nor the buffer-proton equilibria have nonzero ΔC_p values. It should also be noted that the buffer concentration is much higher than the proton uptake/release so that no change in pH occurs upon binding.

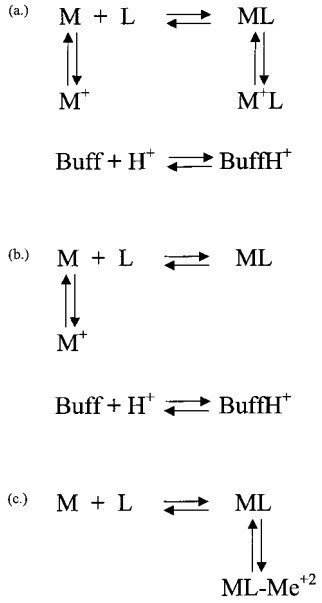
Simulations were performed under two different conditions: open and closed. The open simulation mimics the experimental design in which the pH of the system is adjusted to be the same at each experimental temperature. The closed simulation mimics the experimental design in which the pH is set at 25 °C and allowed to vary with temperature according to the ΔH° and ΔC_p of ionization of the buffer. These two different experimental designs represent holding different variables constant in taking the partial derivative in eq 2. In the open system pH is kept constant, whereas in the closed system the total number of protons is kept constant.

In the closed system, the free proton concentration varies with temperature as follows:

$$[H^+] = [H^+]_R\cdot\exp\left[-\frac{\Delta H_i}{R}\left(\frac{1}{T} - \frac{1}{T_R}\right) + \left(\frac{\Delta C_{p,i}}{R}\right)\left(\frac{T_R}{T} - 1 + \ln\left(\frac{T}{T_R}\right)\right)\right] \quad (5)$$

where $[H^+]_R$ is the proton concentration at the reference temperature, T_R, and T is the temperature of interest, R is the gas constant, and ΔH_i° and $\Delta C_{p,i}$ are the enthalpy and heat capacity of ionization of the buffer, respectively.

Scheme 1: Outline of the Equilibria Linked to the Binding of a Ligand (L) to a Macromolecule (M) Examined in the Simulations[a]



[a] (a) Linked system where a proton can bind both the free and complexed macromolecule and the corresponding uptake or release of protons from the buffer. (b) Linked system where a proton can bind only the free macromolecule and the corresponding uptake or release of protons from the buffer. (c) Linked system where an ion can bind only the ligand−macromolecule complex.

Table 1: Simulation Parameters for a Proton Linked Ligand−Macromolecule Binding Reaction (Scheme 1a)

K_{int}	4×10^{10}
ΔH°_{int}	−2.5 kJ/mol
ΔS°_{int}	195 J/mol/K
ΔC_p	−1.1 kJ/mol/K
pK_a^f	7
pK_a^c	4
ΔH^f_p	−28 kJ/mol
ΔH^c_p	−15 kJ/mol
$pK_{a,buff}$	7.2
ΔH^b_i	37 kJ/mol
$\Delta C_{p,buff}$	−16 J/mol/K
[buffer]	50 mM
T_{ref}	298.15 K

A linked binding system was also simulated where a nonproton ligand (e.g., a metal ion) would only bind to the complexed state of a ligand−macromolecule (see Scheme 1c). The observed binding constant, K_{obs}, is then found as follows:

$$K_{obs} = K_{int}(1 + K_{ion}[ion]) \quad (6)$$

where K_{ion} is the ion binding constant and [*ion*] is the free ion concentration (note that this applies to specific ion-interactions and not generalized salt effects.). The observed enthalpy is defined by the "intrinsic" ligand−macromolecule binding enthalpy, ΔH°_{int}, and the ion-complex binding enthalpy, ΔH°_{ion} as follows:

$$\Delta H^{\circ}_{obs}(T) = \Delta H^{\circ}_{int}(T) + \bar{X}\Delta H^{\circ}_{ion}(T) \quad (7)$$

where $\bar{X}$ is the fractional saturation of ion bound to the ligand−macromolecule complex ($(K_{ion}[ion])/(1 + K_{ion}[ion])$). Simulation parameters are shown in Table 2.

Table 2: Simulation Parameters for an Ion Binding Linked Ligand−Macromolecule Binding Reaction (Scheme 1c)

K_{int}	3.9×10^9
ΔH°_{int}	−10 kJ/mol
ΔS°_{int}	150 J/K/mol
$\Delta C_{p,int}$	−1.0 kJ/K/mol
[ion]	1nM-1mM
ΔG°_{ion}	−34.2 kJ/mol
K_{ion}	1.0×10^6
ΔH°_{ion}	−50 kJ/mol
ΔS°_{ion}	48 J/K/mol
$\Delta C_{p,ion}$	−600 J/K/mol
T_R	298.15 K

RESULTS AND DISCUSSION

Binding simulations were performed in which proton binding was coupled to ligand−macromolecule binding (see Scheme 1a and 1b). If ΔH°_{vH} and ΔH°_{cal} represent the same enthalpy contributions they should agree across the temperature range simulated; however, if ΔH°_{vH} represents the enthalpy independent from linked equilibria, we expect to observe discrepancies. Simulation parameters are defined in Theory. These include the thermodynamic parameters for ligand−macromolecule binding, the binding constants and enthalpy parameters for proton binding, and the thermodynamic parameters for buffer protonation.

Closed System. The simulated experiments show that ΔH°_{cal} and ΔH°_{vH} are equivalent throughout the entire temperature range, as shown in Figure 1a. Therefore, both ΔH°_{cal} and ΔH°_{vH} values represent the enthalpies associated with *all* equilibria shown in Scheme 1a, proton−macromolecule, ligand−macromolecule, and proton−buffer binding. It is important to note that both ΔH°_{cal} and ΔH°_{vH} show dependence on the buffer (due to different enthalpies of ionization) under these experimental conditions as expected for a proton linked system (*17*). The corresponding van't Hoff plot is depicted in Figure 1b. Whereas the first derivative of the van't Hoff plot yields ΔH°_{vH}, the second derivative yields ΔC_p (note the degree of curvature that can be expected for a protein−protein interaction with a negative heat capacity change). As a second test, a simulation was performed in which a proton can bind only a free macromolecule (see Scheme 1b). Similar to the first simulation, ΔH°_{cal} and ΔH°_{vH} were found to be equivalent, regardless of buffer type.

A simulation of ion-linkage (Scheme 1c) provides a third example to test the effects of linked equilibria on ΔH°_{cal} and ΔH°_{vH} values. This example, unlike the previous two, is not dependent on pH in that the linkage comes from an ion binding the ligand−macromolecule complex where the ion binding is taken to be pH-independent. Although ΔH°_{cal} values are expected to have contributions from both ligand−macromolecule binding and ion−complex binding, ΔH°_{cal} and ΔH°_{vH} values are identical (Figure 2a), indicating that the van't Hoff enthalpy also includes contributions from both binding equilibria. The corresponding van't Hoff plot is shown in Figure 2b.

As with enthalpy comparisons, calorimetric heat capacity changes, $\Delta C_{p,cal}$, will match those heat capacity changes determined by the van't Hoff method, $\Delta C_{p,vH}$. However, one

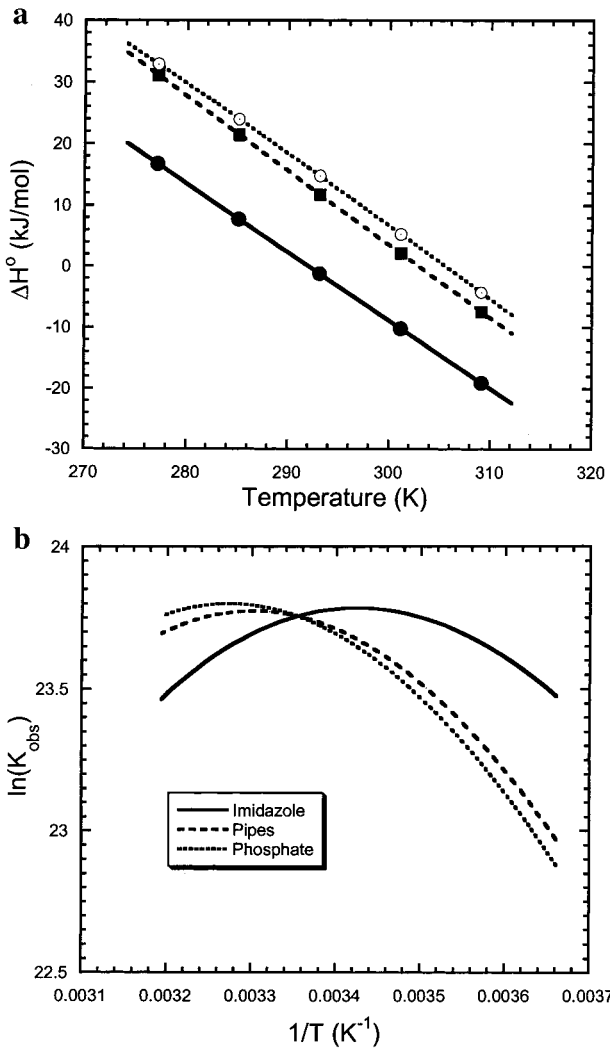


FIGURE 1: (a) Temperature dependence of the calorimetric and van't Hoff enthalpies for a proton linked system (Scheme 1a) using three different buffers: (●) ΔH°_{cal} and (—) ΔH°_{vH} with Imidazole; (■) ΔH°_{cal} and (- - -) ΔH°_{vH} with Pipes; and (○) ΔH°_{cal} and (·····) ΔH°_{vH} with phosphate. (b) The corresponding van't Hoff plot for a proton linked ligand−macromolecule binding equilibrium (Scheme 1a). See legend inset for buffer description.

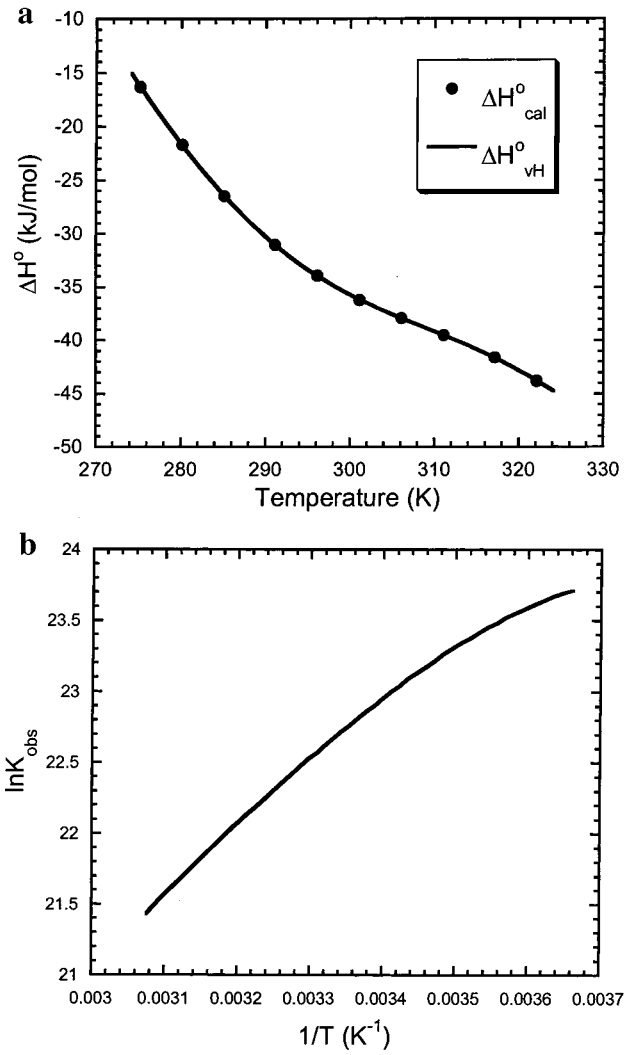


FIGURE 2: (a) Temperature dependence of the calorimetric and van't Hoff enthalpies for an ion linked ligand−macromolecule binding system (Scheme 1c). See legend inset for description. (b) Corresponding van't Hoff plot for an ion linked ligand−macromolecule system (Scheme 1c).

must pay careful attention when interpreting heat capacity changes under linked systems, such as these three examples, because the presence of linked equilibria will affect the profile of the temperature dependency of the enthalpy and, consequently, the determined ΔC_p values (*18*). The simulation with ion binding to the ligand−macromolecule complex (Scheme 1c) demonstrates the dramatic effect the linked equilibrium can have on the enthalpy profile (Figure 2a). Although less "dramatic," the variance in the temperature profile of the enthalpy is still observed even in the absence of heat capacity terms for ligand and ion binding (Figure 3). These profiles result from conditions in which the fractional saturation of the ion-bound complex is changing over the temperature range studied (e.g., when ion concentrations are close to K_d). Consequently, ΔH° is not a linear function of temperature. The determined ΔC_p values, either through calorimetric or van't Hoff methods, will show dependence on experimental conditions (i.e., the concentration of the linking species). However, if concentrations of the linked species (ions in this example) are increased to saturate the complex throughout the temperature range studied, the ΔH° is linear with temperature (Figure 3b). Under these conditions the observed ΔH° for a given temperature will simply be the sum of the enthalpies of ligand−macromolecule binding and ion−complex binding at that temperature. Similarly, the observed ΔC_p will be the sum of the heat capacity changes for ligand−macromolecule and ion−complex binding (see Figure 4a and 4b). In either linked-equilibria example, calculations of ΔC_p based on changes in polar and apolar surface area (*19*) will not agree with the observed ΔC_p. However, the use of parameters based on changes in polar and apolar surface area can suggest the presence of an additional linked equilibrium such as proton linkage (*18*) or conformational change (*20*) under some circumstances.

These simulations demonstrate that ΔH°_{vH} is equivalent to ΔH°_{cal} for closed systems, regardless of the type or degree of linked equilibrium (e.g., proton linkage, ion binding, or conformational change (*7*)). Therefore, ΔH°_{vH} does not represent the "intrinsic" ligand−macromolecule binding enthalpy, but the overall ΔH° of the reaction when maintain-

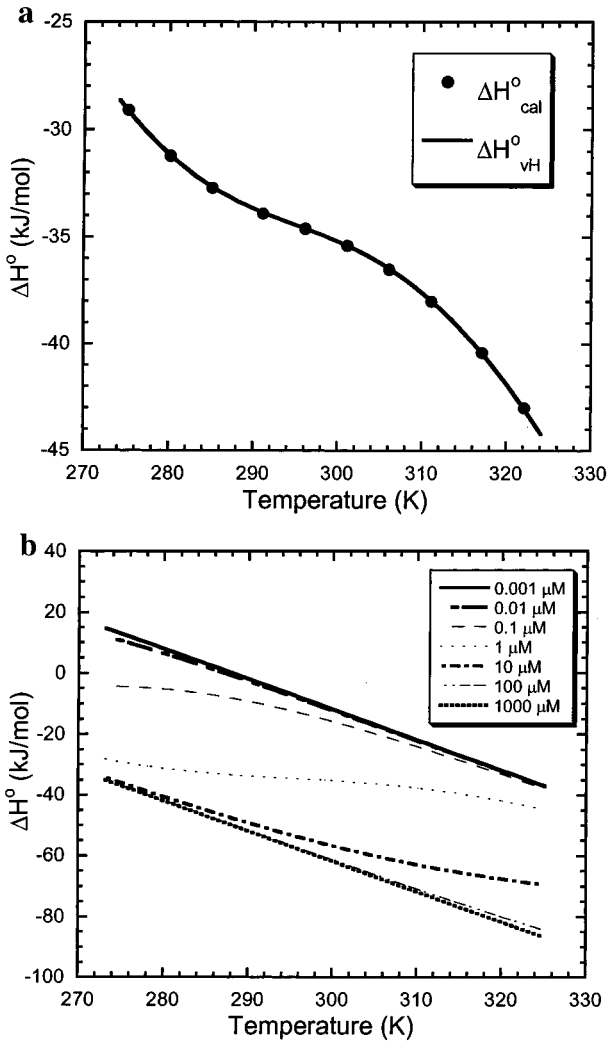


FIGURE 3: (a) Temperature dependence of the calorimetric and van't Hoff enthalpies for an ion−ligand−macromolecule binding equilibria with a ΔC_p of ion binding equal to zero. See legend inset for description. (b) Observed enthalpy dependence on the concentration of the linking ion species. See inset for ion concentrations.

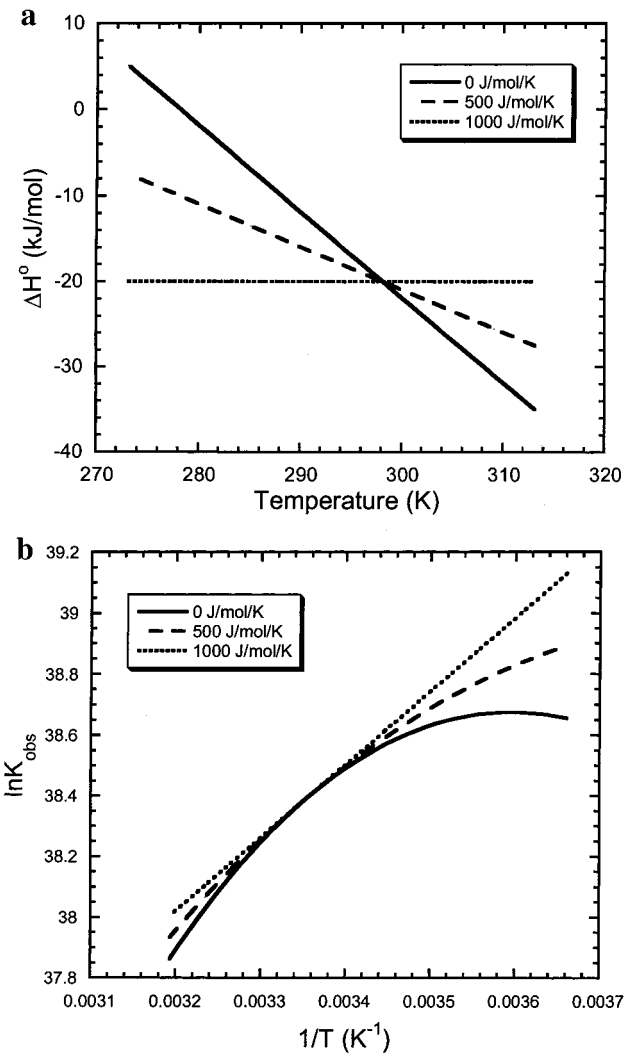


FIGURE 4: (a) Temperature dependence of the enthalpy as a function of different ΔC_p values for the ion binding equilibrium in the ion−ligand−macromolecule binding equilibria system. See inset for ΔC_p values. (b) Effect of various ΔC_p values of the ion binding equilibrium on the van't Hoff plot.

ing a "closed" system. The simulations also provide evidence that there is no theoretical reason to expect ΔH°_{cal} and ΔH°_{vH} to be different, even without prior knowledge of the presence or absence of linked equilibria.

Open System. There are experimental conditions that can result in a genuine discrepancy between ΔH°_{cal} and ΔH°_{vH}. Discrepancies between ΔH°_{cal} and ΔH°_{vH} can arise from the experimental setup. In a proton-linked binding system (e.g., Scheme 1a or 1b) this will occur when pH is adjusted so as to be the same at each temperature. As a consequence, the free proton concentration is held constant (by adjusting the total proton concentration) as the temperature is varied. Therefore, in the van't Hoff analysis both temperature and total proton concentration will vary. As this setup is used in determination of ΔH°_{vH} and $\Delta C_{p,vH}$ (*21, 22*), it is important to understand what reaction these values represent.

The consequence of this scenario under conditions where proton linkage is present is depicted in Figure 5. In this simulation the pH is in a region where the group on the macromolecule involved in the linked proton equilibria exhibits a change in fractional saturation with temperature. The pH is not allowed to vary as the temperature is changed. Consequently, the ligand−macromolecule equilibrium is changing as a function of temperature, while the proton binding equilibria change with temperature but are forced to have the same free proton concentration (the latter would be determined from the thermodynamics of buffer ionization in a closed system). As a result, the van't Hoff enthalpy will lack contributions from the last term of eq 4 which describes the buffer contribution.

The van't Hoff enthalpy under this scenario is described by

$$\Delta H^\circ_{vH}(T) = \Delta H^\circ_{int}(T) - \bar{H}^f \Delta H^f_p + \bar{H}^c \Delta H^c_p \qquad (8)$$

which contains contributions from the enthalpy of ligand−protein binding, ΔH°_{int}, and proton binding to the free and complexed macromolecule, ΔH^f_p and ΔH^c_p, respectively. This is equivalent to the intercept in a plot of ΔH°_{cal} vs ΔH°_{ion} of the buffer. In other words, it is the ΔH° which would be observed calorimetrically in a buffer with ΔH°_{ion} equal to zero. Figure 5 shows that for the open system there is a dependence of the calorimetric enthalpy on the enthalpy of ionization of the buffer, whereas the van't Hoff enthalpy

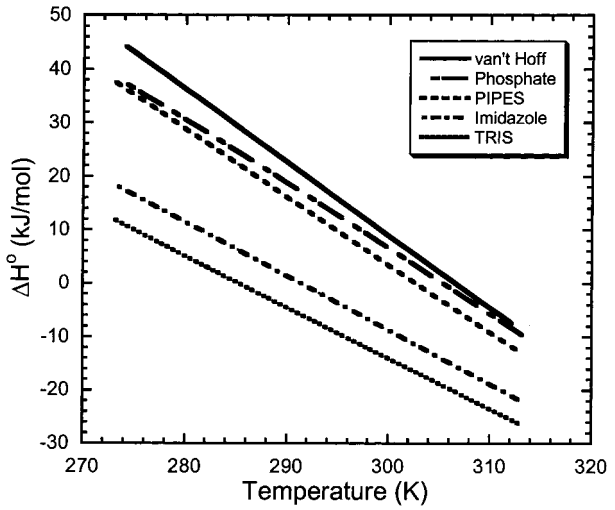


FIGURE 5: Comparison between the van't Hoff and calorimetric enthalpies determined in various buffers under "open conditions". The solid line represents the van't Hoff enthalpy determined in any of the four buffers. The dashed lines represent calorimetric enthalpies for each buffer.

is buffer independent (a simulation with a buffer enthalpy of ionization equal to zero shows no discrepancy between ΔH°_{vH} and ΔH°_{cal}). Thus, an "open" experimental design is a useful means of obtaining the "intercept" ΔH° value without using multiple buffers.

The $\Delta C_{p,cal}$ and $\Delta C_{p,vH}$ values will also disagree in this situation (as can be seen by the different temperature dependencies of the enthalpies). The $\Delta C_{p,vH}$ under these conditions will be defined by the temperature dependence of eq 8 (see ref *16*):

$$\Delta C_{p,vH} = \Delta C_{p,\mathrm{int}} + \frac{\partial N_{H^+}}{\partial T}\Delta H_p^f + N_{H^+}\Delta C_{p,p}^f + \frac{\partial \bar{H}^c}{\partial T}(\Delta H_p^c - \Delta H_p^f) + \bar{H}^c(\Delta C_{p,p}^c - \Delta C_{p,p}^f) \quad (9)$$

where $\Delta C_{p,\mathrm{int}}$ is the intrinsic heat capacity change for ligand−macromolecule binding, N_{H^+} is the number of protons released from the buffer, $\Delta C_{p,p}^f$ is the heat capacity change for protonation of the free macromolecule, and $\Delta C_{p,p}^c$ is the heat capacity change for protonation of the complexed macromolecule.

As demonstrated in Figure 5, the differences between ΔH°_{cal} and ΔH°_{vH} can be quite dramatic. Therefore, caution must be used when designing experimental strategies to obtain van't Hoff enthalpies. This scenario may actually be exploited by those interested in using the van't Hoff method to obtain enthalpy values separated from contributions of buffer equilibria. However, if one is interested in dissecting the proton linkage contributions from the ligand−macromolecule binding enthalpy using the van't Hoff method, one would need also to use the "closed" experimental design and analyze ΔH°_{vH} as a function of pH as described previously for ITC (*17*). It must be noted, however, that this will be experimentally accessible only with proton linkage and not with linkage due to folding or ion binding.

These simulations illustrate that the ΔH° measured by using the van't Hoff method will depend on the experimental design. If the ΔH° that would be observed in a buffer with no ionization enthalpy is desired, then the pH should be adjusted so as to be the same at each temperature; however, this will not be equal to ΔH°_{cal} under identical conditions. If the intrinsic ΔH° of binding is desired, then the pH should not be adjusted within a temperature series (so as to be equivalent to ΔH°_{cal}) and experiments at different pH values and in buffers with differing ionization enthalpies will be required as in an ITC experiment (*17*).

Other Scenarios for Discrepancies. Considering experimenter intervention can lead to calorimetric-van't Hoff discrepancies in the case of proton linkage, other cases exist that can lead to discrepancies. Assuming one takes into account the considerations discussed above (i.e., allowing the system to equilibrate with temperature), maintaining equilibrium conditions can still be problematic. One example is the presence of irreversible aggregation when working with proteins, which occurs in a temperature- and concentration-dependent manner. The aggregating species would introduce a new species that would not reequilibrate with changing temperature, and would negate the van't Hoff analysis. Here, if one had access to both ΔH°_{cal} and ΔH°_{vH}, discrepancies between calorimetric and van't Hoff enthalpies can indicate the presence of experimental artifacts. Additional common problems have been discussed previously (*7*) and include problems in data analysis, such as the assumption that a van't Hoff plot is linear, and error propagation.

Overall, as long as all variables are allowed to fluctuate with temperature, no discrepancy between calorimetric and van't Hoff enthalpies will be observed. Simulations of the thermodynamics of various linked binding systems demonstrate that the presence of linked equilibria do not affect this agreement even though the presence of linked equilibria can result in "nonconventional" enthalpy profiles, making data analysis nontrivial. Finally, large ΔC_p contributions can arise from the presence of coupled equilibria so that this parameter will not necessarily be reflective of changes in solvation of protein−ligand surfaces.

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