Evaluation of Linked Protonation Effects in Protein Binding Reactions Using Isothermal Titration Calorimetry

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ABSTRACT A theoretical development in the evaluation of proton linkage in protein binding reactions by isothermal titration calorimetry (ITC) is presented. For a system in which binding is linked to protonation of an ionizable group on a protein, we show that by performing experiments as a function of pH in buffers with varying ionization enthalpy, one can determine the pK_a's of the group responsible for the proton linkage in the free and the liganded states, the protonation enthalpy for this group in these states, as well as the intrinsic energetics for ligand binding ($\Delta H°$, $\Delta S°$, and ΔC_p). Determination of intrinsic energetics in this fashion allows for comparison with energetics calculated empirically from structural information. It is shown that in addition to variation of the ligand binding constant with pH, the observed binding enthalpy and heat capacity change can undergo extreme deviations from their intrinsic values, depending upon pH and buffer conditions.

INTRODUCTION

The binding of ligands to proteins is of central importance in biology. Generally, these interactions show some degree of pH dependence, reflecting the linkage between the binding of ligand and the binding of protons (proton linkage). This linkage is quantitated as a change in the ligand binding constant with pH, or as a change in the proton affinity (i.e., pK_a) of an ionizable group in the protein upon ligand binding. In determining proton linkage, one typically measures the affinity constant for the binding of the ligand at a number of pH values and calculates the pK_a of the protein in the free and liganded states. However, this is problematic if the ligand binding constant is too large to be readily measured or if the pK_a shift is small.

An alternative approach to measuring affinity constants at different pH values is to examine enthalpic effects using isothermal titration calorimetry (ITC). ITC measures the heat effect when an aliquot of ligand solution is added to a protein solution (Wiseman et al., 1989; Freire et al., 1990). When the binding is of moderate affinity, ITC can be used to determine an observed binding constant, K_{obs}, and an observed enthalpy change, $\Delta H°_{obs}$. When the binding is of high affinity ($>10^8$), $\Delta H°_{obs}$ can still be determined with high precision, even though K_{obs} cannot be determined. The power of ITC in evaluating proton linkage lies in the contribution of the linkage effects to $\Delta H°_{obs}$.

Proton linkage has occasionally been exploited in ITC studies. Strong proton linkage has been used to determine affinity constants that are too high to be determined at physiological pH (Doyle et al., 1995). In several studies the number of protons exchanged upon binding has been determined at one or more pH values (Murphy et al., 1993; Takahashi and Fukada, 1985; Kresheck et al., 1995; Gómez and Freire, 1995), and occasionally, the change in pK_a of an ionizable group has been determined (Eftink et al., 1983; Takahashi and Fukada, 1985). In this paper we describe the theoretical basis for analyzing proton linkage using ITC measurements and illustrate the range of effects through simulations (see also Yung and Trowbrige, 1980).

Additionally, there is significant interest in correlating experimentally measured binding energetics with energetics calculated empirically from structural information (Bhat et al., 1994; Connelly et al., 1990, 1994; Connelly and Thomson, 1992; Kresheck et al., 1995; Murphy et al., 1993, 1995; Spolar and Record, 1994; Ysern et al., 1994). We show here how proton linkage can contribute to experimental energetics and how the intrinsic values can be determined from ITC measurements for comparison with empirical calculations. In doing so, we demonstrate how ITC can exploit proton linkage effects to determine the enthalpies of protonation and pK_a changes that occur upon binding.

THEORY

Determination of proton linkage

We begin with a simple picture for linked binding as illustrated in Scheme 1, in which a protein can bind a single ligand and a single proton:

$$\begin{array}{ccccc} & & K_{int} & & \\ & M & \leftrightarrow & ML & \\ K_p^f & \updownarrow & & \updownarrow & K_p^c \\ & M^+ & \leftrightarrow & ML^+ & \end{array} \qquad (1)$$

Here M represents the unprotonated, unligated protein; M^+ represents the protonated, unligated protein; L represents the ligand; and ML and ML^+ represent the unprotonated and protonated complexes. Ligand binding reactions are shown in the horizontal direction and protonation reactions are shown in the vertical direction. K_{int} is the binding constant for the unprotonated protein, and K_p^f and K_p^c are the

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proton binding constants for the free and liganded forms of the protein (equal to 10^{pKa} of the ionizing group).

The observed ligand binding constant, K_{obs}, at any proton activity, a_{H+} (equal to 10^{-pH}), is given as

$$K_{obs} = K_{int}\frac{1 + K_p^c a_{H^+}}{1 + K_p^f a_{H^+}}, \quad (2)$$

so that the proton linkage can be viewed as the change in the proton affinity of the protein that occurs upon ligand binding. Because of this change in proton affinity, protons will either be absorbed or released upon binding of the ligand.

The fractional saturation of protons in the free protein, $\bar{H}^f$, is given as

$$\bar{H}^f = \frac{K_p^f a_{H^+}}{1 + K_p^f a_{H^+}}. \quad (3)$$

Likewise for the protein in complex with ligand:

$$\bar{H}^c = \frac{K_p^c a_{H^+}}{1 + K_p^c a_{H^+}}. \quad (4)$$

The change in the number of protons bound by the protein upon binding of the ligand is the difference between these:

$$N_{H^-} = \bar{H}^c - \bar{H}^f = \frac{K_p^c a_{H^+}}{1 + K_p^c a_{H^+}} - \frac{K_p^f a_{H^+}}{1 + K_p^f a_{H^+}}. \quad (5)$$

Determination of N_{H+} at a minimum of two pH values is thus theoretically sufficient to determine the pK_a in the free and complexed protein, even when the ligand affinity is too tight to be measured.

The value of N_{H+} can be determined using ITC because it contributes to the measured binding enthalpy, ΔH°_{obs}; because there is a change in the number of bound protons and because those protons are either taken up or released by the buffer, the measured binding enthalpy in an ITC experiment is

$$\Delta H^\circ_{obs} = \Delta H^\circ_0 + N_{H^+}\Delta H_i^b, \quad (6)$$

where ΔH_i^b is the ionization enthalpy of the buffer and ΔH°_0 is the enthalpy that would be measured in a buffer with ΔH_i^b equal to zero. For a series of ΔH°_{obs} measured at constant pH in multiple buffers, a plot of ΔH°_{obs} versus ΔH_i^b will yield a slope equal to N_{H+} and an intercept of ΔH°_0 (e.g., Takahashi and Fukada, 1985; Murphy et al., 1993; Kresheck et al., 1995; Eftink and Biltonen, 1980).

Enthalpic effects

The term ΔH°_0 in Eq. 6 is not equal to the intrinsic binding enthalpy, ΔH°_{int} (i.e., the ΔH° corresponding to K_{int} in Scheme 1), but contains contributions from protonation. This contribution is given explicitly from Scheme 1 as

$$\Delta H^\circ_0 = \Delta H^\circ_{int} - \bar{H}^f\Delta H_p^f + \bar{H}^c(\Delta H_p^f + \delta\Delta H_p). \quad (7)$$

Here ΔH_p^f is the enthalpy of protonation of the free protein and $\delta\Delta H_p$ is the change in this quantity that occurs upon binding of the ligand. Both $\bar{H}^f$ and $\bar{H}^c$ are dependent on pH according to Eqs. 3 and 4. Substitution of Eq. 5 into Eq. 7 gives

$$\Delta H^\circ_0 = \Delta H^\circ_{int} + N_{H^+}\Delta H_p^f + \bar{H}^c\delta\Delta H_p. \quad (8)$$

Thus, determination of ΔH°_0 at a third pH value, in addition to the determinations of ΔH°_{obs} versus ΔH_i^b noted above, is theoretically sufficient to determine the intrinsic binding enthalpy, the protonation enthalpy of the free protein, and the protonation enthalpy of the liganded protein. Substitution of Eq. 8 into Eq. 6 yields an expression for the measured binding enthalpy at any given pH and ΔH_i^b:

$$\Delta H^\circ_{obs} = \Delta H^\circ_{int} + N_{H^+}\Delta H_p^f + \bar{H}^c\delta\Delta H_p + N_{H^+}\Delta H_i^b. \quad (9)$$

Equation 9 describes a three-dimensional surface relating ΔH°_{obs} to pH and ΔH_i^b. Measurements of ΔH°_{obs} as a function of pH and ΔH_i^b can be fit globally to Eq. 9 to determine the pK_a in the free protein, the pK_a in the complexed protein, ΔH°_{int}, ΔH_p^f, and $\delta\Delta H_p$.

Heat capacity effects

Another important experimental parameter that can be obtained by ITC is the change in heat capacity upon ligand binding, ΔC_p. This parameter is of particular interest because it reflects changes in hydration of protein surfaces (Sturtevant, 1977; Baldwin, 1986; Gill and Wadsö, 1976; Murphy et al., 1990; Dill, 1990; Makhatadze and Privalov, 1990). The observed ΔC_p is given as the temperature derivative of ΔH°_{obs} (Eq. 6):

$$\Delta C_{p,obs} = \frac{\partial\Delta H^\circ_{obs}}{\partial T} = \Delta C_{p,0} + \frac{\partial N_{H^+}}{\partial T}\Delta H_i^b + N_{H^+}\Delta C_{p,i}^b. \quad (10)$$

Here, $\Delta C_{p,0}$ is the temperature derivative of ΔH°_0 at the specified pH, and $\Delta C_{p,i}^b$ is the heat capacity change for ionization of the buffer. To obtain the intrinsic ΔC_p, $\Delta C_{p,0}$ must be analyzed explicitly from Eq. 8 as

$$\Delta C_{p,0} = \Delta C_{p,int} + \frac{\partial N_{H^+}}{\partial T}\Delta H_p^f + N_{H^+}\Delta C_{p,p}^f + \frac{\partial\bar{H}^c}{\partial T}\delta\Delta H_p + \bar{H}^c\delta\Delta C_{p,p}, \quad (11)$$

where $\Delta C_{p,p}^f$ is the heat capacity change for protonation of the free protein, and $\delta\Delta C_{p,p}$ is the change in this value that occurs upon binding. Both equations 10 and 11 contain the temperature derivative of N_{H+}, which can be evaluated from Eq. 5 at constant proton activity from Eq. 8 as

$$\frac{dN_{H^+}}{dT} = \frac{\partial\bar{H}^c}{\partial T} - \frac{\partial\bar{H}^f}{\partial T} = \left(\frac{(\Delta H_p^f + \delta\Delta H_p^f)}{RT^2}\frac{K_p^c a_{H^+}}{(1 + K_p^c a_{H^+})^2}\right) - \left(\frac{\Delta H_p^f}{RT^2}\frac{K_p^f a_{H^+}}{(1 + K_p^f a_{H^+})^2}\right), \quad (12)$$

where R is the gas constant and T is the absolute temperature. The other term that needs to be evaluated in Eq. 11 is the

temperature derivative of $\bar{H}_c$, which is the first term on the right side of Eq. 12. It is of interest that Eqs. 11 and 12 have an explicit temperature dependence that will contribute to ΔC_p whenever there is proton linkage. Note also from Eqs. 10–12 that pH plays a prominent role in the measured value of ΔC_p, and that substitution of Eqs. 11 and 12 into Eq. 10 yields an expression for $\Delta C_{p,obs}$ as a function of pH and ΔH_b^i.

Entropic effects

Although it is possible to evaluate the linkage effects on the measured entropy change directly, the intrinsic value of the entropy change, ΔS°_{int}, may be readily obtained from the value of ΔG°_{int} (calculated from K_{int}) and the value ΔH°_{int} by using the standard relationship

$$\Delta S^\circ_{int} = \frac{\Delta H^\circ_{int} - \Delta G^\circ_{int}}{T}. \quad (13)$$

The above equations can be used to obtain all of the requisite thermodynamic parameters implicit in Scheme 1 from experimental ITC data in a variety of buffers, over a range of pH values. Although these equations have been derived for a single ionizable site on the protein, the treatment can be extended to multiple sites by including the appropriate binding polynomials, their applicable enthalpic effects, and temperature derivatives (Wyman and Gill, 1990).

RESULTS AND DISCUSSION

Simulations were performed to illustrate the experimental results which would be obtained in a protein-linked ligand binding study performed by ITC. Parameters used in the simulations are listed in Table 1 and represent a "typical" protein-ligand interaction in which the protonation of a histidine side chain is linked to ligand binding. Unless otherwise noted, values of ΔH_i^b and $\Delta C_{p,i}^b$ were selected to represent an imidazole buffer.

pH dependency of the binding constant

Fig. 1 details the behavior of the measured binding constant, K_{obs}, with pH, as given by Eq. 2. Note that the measured value would only be equal to the intrinsic value at high pH.

TABLE 1 Values for the thermodynamic parameters used in generating Figs. 1–9

Parameter	Value
K_{int}	10^8 M^{-1}
$pK_{a,f}$	7
$pK_{a,c}$	5.5
ΔH°_{int}	−10 kJ mol^{-1}
ΔH^f_p	−30 kJ mol^{-1}
$\delta\Delta H_p$	5 kJ mol^{-1}
$\Delta C_{p,int}$	−0.3 kJ K^{-1} mol^{-1}
$\Delta C^f_{p,p}$	−16 J K^{-1} mol^{-1}
$\delta\Delta C_{p,p}$	−10 J K^{-1} mol^{-1}
$\Delta C^b_{p,i}$	16 J K^{-1} mol^{-1}

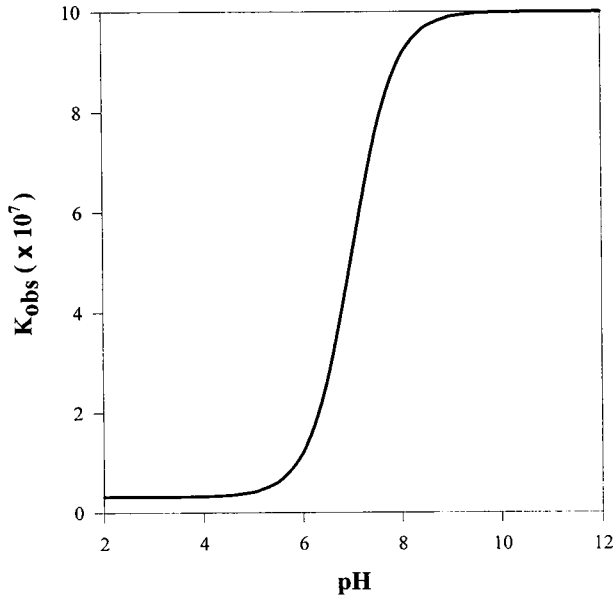

FIGURE 1 pH dependency of the observed ligand binding calculated according to Eq. 2 using the values in Table 1. The observed binding constant is equal to the intrinsic binding constant only above pH 9.

It is possible to exploit this behavior experimentally: consider a proton-linked system that experiences a large pK_a shift upon ligand binding, yet whose intrinsic binding constant is too large to measure directly. If the pK_a values in the free and complexed protein can be determined, it may be possible to measure the binding constant at a pH where it is experimentally accessible, and then calculate the intrinsic binding constant. The method for determining pK_a changes outlined here readily lends itself to such an approach (see also Doyle et al., 1995).

Release/absorption of protons upon binding

Fig. 2 shows simulated ΔH°_{obs} versus ΔH_i^b plots for pH values of 5, 6, and 7. The lines are described by Eq. 6, with slope equal to N_{H+}, the number of protons released by the buffer upon binding, and intercept ΔH°_0, the enthalpy change that would be measured at that pH in a buffer with ΔH_i^b equal to zero.

The lack of a common point of intersection for the lines reflects the presence of $\delta\Delta H_p$. With $\delta\Delta H_p$ equal to zero, plots of ΔH°_{app} versus ΔH_i^b at any pH would all intersect where ΔH_i^b is equal to and opposite the protonation enthalpy of the protein. Such a case is unlikely for amine-type groups with large values of ΔH_i^b; it is more likely for carboxylates, which have small values of ΔH_i^b.

Fig. 3 shows the variation of N_{H+} with pH for pK_a changes from 7 to 5.5, 6.5, and 6.75. As discussed above, determinations of N_{H+} at a minimum of two pH values can be analyzed to determine the pK_a for the ionizing group in the free and the complexed protein (Eq. 5). Determination of N_{H+} at the pH where it is a maximum can help to establish if more than one ionizable group contributes to the

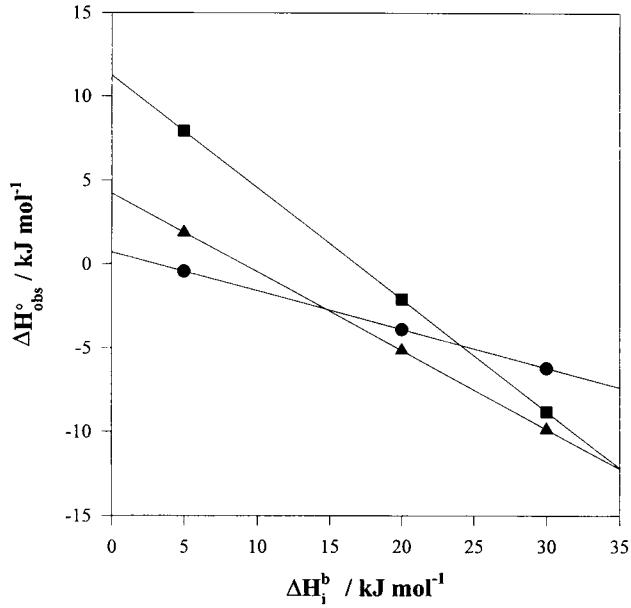

FIGURE 2 ΔH°_{obs} versus ΔH^b_i at pH 5 (●), pH 6 (▲), and pH 7 (■) calculated using the values in Table 1. Slopes are equal to N_{H+} and intercepts are equal to ΔH°_0. The lack of a common point of intersection for the three lines reflects the non-zero value of $\delta\Delta H_p$.

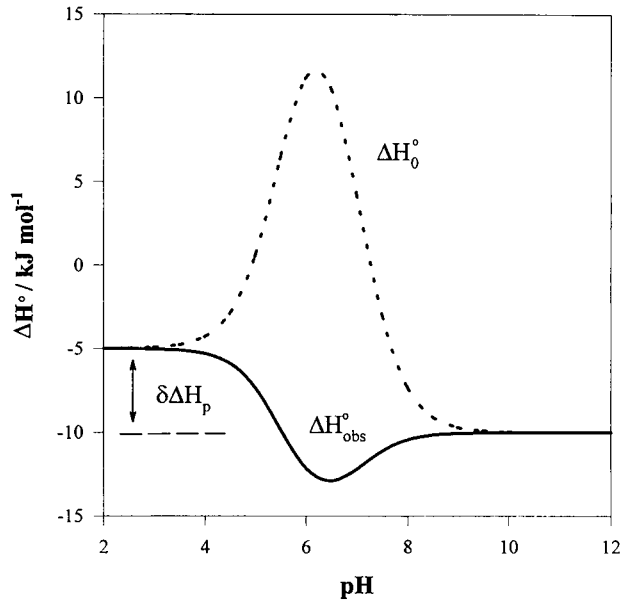

FIGURE 4 ΔH°_{obs} (——) and ΔH°_0 (– – –) as a function of pH calculated from the values in Table 1 and with ΔH^b_i set to 35 kJ/mol. The difference in ΔH°_{obs} between low and high pH is equal to $\delta\Delta H_p$.

proton linkage. As N_{H+} represents the slopes of ΔH°_{obs} versus ΔH^b_i (Fig. 2), sensitivity in determining pK_a's is limited primarily by the availability of buffers with a range of ΔH^b_i at desired pH values.

pH and buffer dependency of ΔH°_{obs}

Fig. 4 details the variation of ΔH°_{obs} and ΔH°_0 with pH at constant ΔH^b_i (Eqs. 8 and 9; see Table 1 and figure legend for values). As with the binding constant, the intrinsic binding enthalpy is only measured at high pH values, and ΔH°_{obs} can deviate markedly from ΔH°_{int}. Fig. 5 shows the contributions of the second and third term in Eq. 7, and the final term in Eq. 6, to ΔH°_{obs}. The shape of the ΔH°_0 curve between pH 3 and 9 is largely due to the higher proton

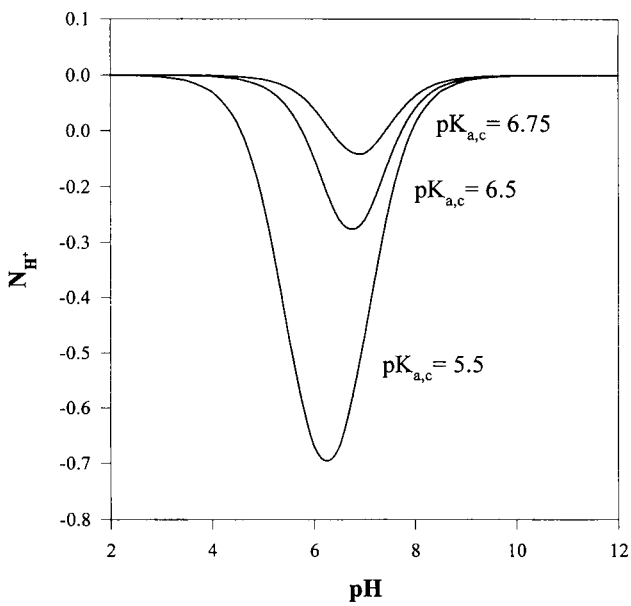

FIGURE 3 N_{H+} versus pH for pK_a shifts from 7 to 5.5, 6.5, and 6.75 calculated using the values in Table 1. Any point along the curve represents slopes of ΔH°_{obs} versus ΔH^b_i at that pH (see Fig. 2).

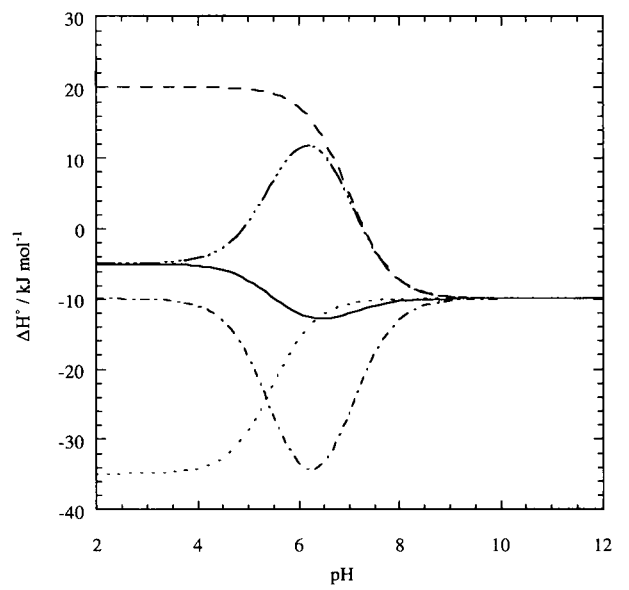

FIGURE 5 Contributions of protonation of the free (– – –) and complexed (·····) states of the protein and of the protonation of the buffer (– · –) to ΔH°_0 (– ··· –) and ΔH°_{obs} (——). The first three lines are calculated, respectively, as the second and third terms of Eq. 7, and the final term of Eq. 6 using the values in Table 1 and with ΔH^b_i set to 35 kJ/mol.

saturation of the free protein relative to the complex. The value of $\delta\Delta H_p$ does not have a large influence in this region, but does dictate the value of the low pH intercept, such that the difference in ΔH°_{obs} between high and low pH is $\delta\Delta H_p$.

The final term in Eq. 6 offsets the influence of ΔH°_0, with the size (and sign) of the "dip" in ΔH°_{int} strongly dependent upon ΔH^b_i. This is underscored in Fig. 6, which shows ΔH°_{obs} as a function of pH and ΔH^b_i. Along the pH axis, the curves represent ΔH°_{obs} as shown in Fig. 4, and along the ΔH^b_i axis the slopes represent N_{H+}, as shown in Fig. 2.

Experiments can be performed in buffers of low ΔH^b_i (for example phosphate, acetate, cacodylate) in an attempt to minimize the protonation effect. However, with a ΔH^b_i of zero, ΔH°_{obs} is actually ΔH°_0, which contains contributions from the protonation enthalpies of the free and complexed protein and the magnitude of the pK_a shift, as shown above (Eq. 8). As demonstrated by Figs. 5 and 6, this contribution can be quite large. If the group responsible for the proton linkage is known, experiments can be performed in a buffer in which the magnitude of ΔH^b_i is close or equal to the protonation enthalpy of the free protein (ΔH^f_p), i.e., a matched buffer. However, even in the case where a buffer is available in which ΔH^b_i exactly offsets ΔH^f_p, there are still contributions from $\delta\Delta H_p$. This is illustrated by Fig. 7, which shows ΔH°_{obs} as a function of pH and $\delta\Delta H_p$, but with ΔH^b_i equal to and opposite ΔH^f_p. Again, the effects can be quite large, and ΔH°_{obs} is only equal to ΔH°_{int} when $\delta\Delta H_p$ is zero.

pH and buffer dependency of $\Delta C_{p,obs}$

The complex variation of $\Delta C_{p,obs}$ with pH is described by Eqs. 10–12 and is shown in Fig. 8 at a constant ΔH^b_i. An intriguing characteristic of $\Delta C_{p,obs}$ is the extrema that occur at pH values equal to the pK_a's of the free and complexed

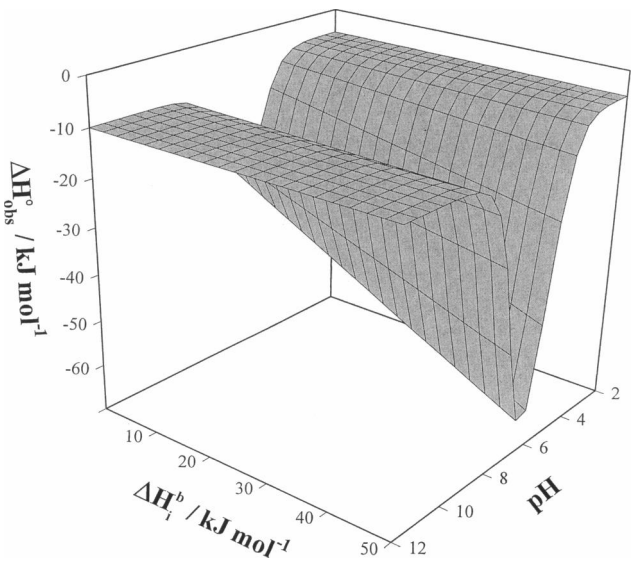

FIGURE 6 ΔH°_{obs} as a function of pH and ΔH^b_i calculated from the values in Table 1. Curves along the pH axis represent ΔH°_{obs} as shown in Fig. 4, and slopes along the ΔH^b_i represent N_{H+} as shown in Fig. 2.

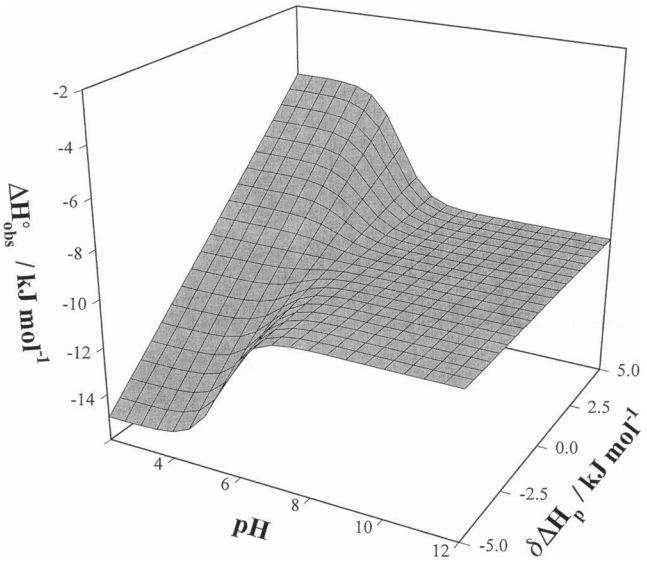

FIGURE 7 ΔH°_{obs} as a function of $\delta\Delta H_p$ and pH calculated from the values in Table 1 and with ΔH^b_i set to 10 kJ/mol (i.e., a matched buffer). ΔH°_{obs} is only equal to ΔH°_{int} when $\delta\Delta H_p$ is zero.

protein. As with ΔH°_{obs}, the shape of the curve is the result of the summation of nearly opposing terms (the second terms of Eqs. 10 and 11), with the imbalance due to the magnitude of ΔH^b_i (second term in Eq. 10). Furthermore, the difference in $\Delta C_{p,obs}$ between high and low pH reflects $\delta\Delta C_{p,p}$. The value of $\Delta C^b_{p,i}$ does not largely influence $\Delta C_{p,obs}$ when changes in $\Delta C^b_{p,i}$ are made with reasonable, concomitant changes in ΔH^b_i (not illustrated). Generally, buffers with large values of ΔH^b_i have small values of $\Delta C^b_{p,i}$,

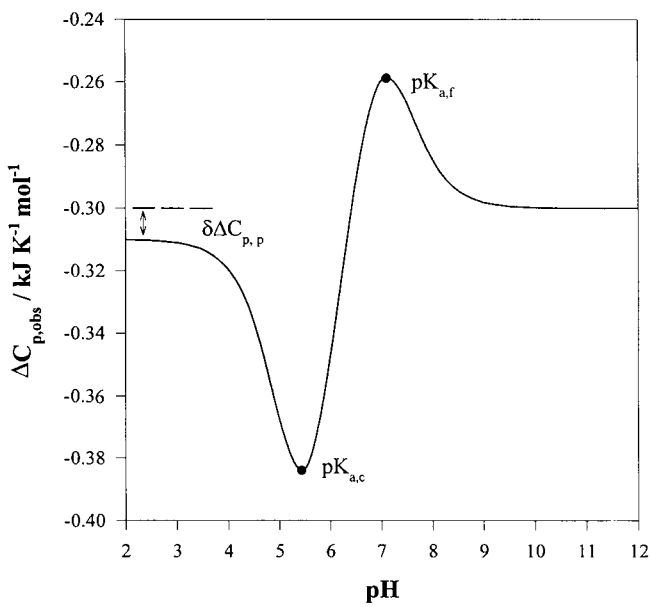

FIGURE 8 $\Delta C_{p,obs}$ as a function of pH calculated from the values in Table 1 and with ΔH^b_i set to 35 kJ/mol. The minimum and maximum occur at pH values equal to the values of $pK_{a,c}$ and $pK_{a,f}$. The difference in $\Delta C_{p,obs}$ between low and high pH is equal to $\delta\Delta C_{p,p}$.

and vice versa (Christensen et al., 1976). Finally, note that we have used an abnormally large value of $\delta\Delta C_{p,p}$ to demonstrate its effect. Fig. 9 illustrates $\Delta C_{p,obs}$ as a function of pH and ΔH_i^b. As with ΔH°_{obs}, the choice of buffer and pH can dramatically influence $\Delta C_{p,obs}$. Although by performing an appropriate number of determinations of $\Delta C_{p,obs}$ versus pH will allow one to determine a value for $\delta\Delta C_{p,p}$, it may be safe to assume a value of zero.

CONCLUSIONS

We have outlined a calorimetric method for determining proton linkage and its contributions to observed thermodynamics in ligand binding reactions. Although the simulations are for one linked proton-binding site, they can be readily extended to two or three sites by using appropriate binding polynomials. In more complicated cases, where many sites are involved, an analytical treatment may not be possible. In such cases a more general treatment, such as that of Doyle et al. (1995), may be required; however, determination of intrinsic binding energetics in such cases is problematic.

By performing experiments as a function of pH and buffer ionization enthalpy, one can directly determine the pK_a's of the group responsible for the proton linkage in the free and the liganded protein, the protonation enthalpy for this group in the free and the liganded protein, and the intrinsic energetics for ligand binding. Although such information cannot be used to identify the proton-binding site on the protein unambiguously, the magnitude of ΔH_p^f can suggest likely candidates. As noted previously, amine groups generally have large values of ΔH_p^f, whereas carboxyl groups generally have small values.

Determination of intrinsic energetics in this manner allows for direct comparison with those calculated empirically from structural information. Finally, simulations demonstrate that such proton linkage can dramatically influence observed thermodynamic values, so that care must be taken in the interpretation of data from such systems.

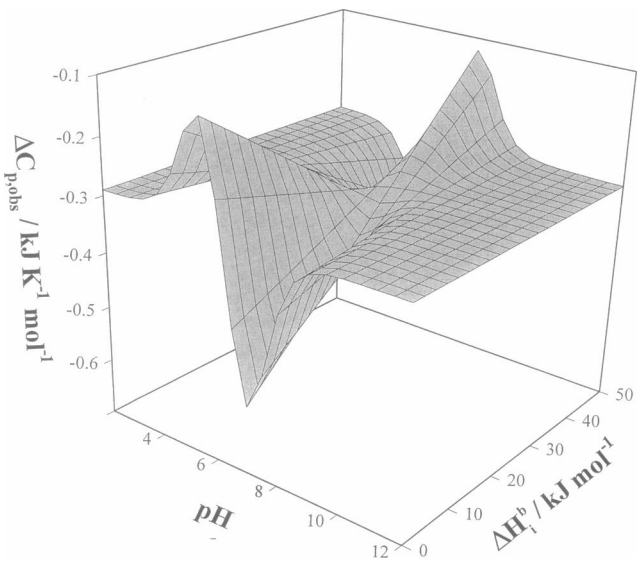

FIGURE 9 $\Delta C_{p,obs}$ as a function of pH and ΔH_i^b calculated using the values in Table 1.

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