Calorimetric Investigation of Proton Linkage by Monitoring both the Enthalpy and Association Constant of Binding: Application to the Interaction of the Src SH2 Domain with a High-Affinity Tyrosyl Phosphopeptide†

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ABSTRACT: The binding of Src homology 2 (SH2) domains to tyrosyl phosphopeptides depends on electrostatic interactions between the phosphotyrosine and its binding site. To probe the role of these interactions, we have used isothermal titration calorimetry to study the pH dependence of the binding of the SH2 domain of the Src kinase to a high-affinity tyrosyl phosphopeptide. Two independent approaches were employed. In a first series of experiments that focused on determining the peptide's association constant between pH 5.0 and 9.0, two ionizable groups were characterized. One group, with free and bound pK_as of 6.2 and 4.4, respectively, could be identified as the phosphate in the phosphotyrosine while the other group, with free and bound pK_as of 8.2 and 8.5, respectively, could be only tentatively assigned to a cysteine in the phosphotyrosine binding pocket. Further information on the linkage between peptide binding and protonation of the phosphotyrosine was obtained from a second series of experiments, which focused on determining the peptide binding enthalpy at low values of pH in several buffers with different ionization enthalpies. These data provided free and bound pK_a values for the phosphotyrosine identical to those derived from the first series of experiments, and hence demonstrated for the first time that the two approaches provide identical information regarding proton linkage. In addition, the second series of experiments also determined the intrinsic enthalpy of binding of both the protonated and deprotonated phosphate forms of the peptide. These two sets of experiments provided a complete energetic profile of the linkage between phosphate ionization and peptide binding. From this profile, it was determined that the PO_3^{2-} form of the peptide binds 2.3 kcal mol^{-1} more favorably than the PO_3H^{1-} form due entirely to a more favorable entropy of binding.

Src homology 2 (SH2) domains are protein modules of ~100 residues found in proteins involved in signal transduction downstream of various cell membrane receptors (*1*, *2*). Their function is to bind sites of tyrosine phosphorylation in cellular proteins, thereby initiating a cascade of events that eventually leads to altered patterns of gene expression (*3*). The central role of SH2 domains in many potentially oncogenic cell signaling processes make these domains an attractive target for anticancer pharmaceuticals (*4*).

SH2 domains of the Src family of protein tyrosine kinases have been extensively studied both structurally and biochemically. The molecular basis of the interaction of these SH2 domains with phosphopeptides has been determined from the crystal structures of several complexes (*5*−*10*). These studies have shown that the phosphotyrosine in the peptide ligand binds within a positively charged pocket of the SH2 domain. The three residues C-terminal to the phosphotyrosine contribute most of the other contacts observed between the phosphopeptide and the protein. However, the extent to which these residues interact with the surface of the binding site is dependent on the presence of a bulky hydrophobic residue, such as Ile, at the +3 position C-terminal to the phosphotyrosine (*6*).

Recently, the role of the peptide residue at the +3 position C-terminal to the phosphotyrosine in high-affinity binding to the SH2 domain of the Src kinase (Src SH2 domain) was examined (*11*). By systematically substituting this position with various hydrophobic side chains, it was shown that high-affinity binding is only partially determined by the interactions between the +3 residue in the peptide and the pocket into which the +3 residue in the peptide inserts. Surprisingly, the thermodynamic parameter that most distinguished high- versus low-affinity binding was $\Delta H°$, suggesting that specific hydrogen bonding and van der Waals interactions, rather than hydrophobic interactions, play a crucial role in promoting high-affinity binding.

While the contribution of the +3 residue to the energetics of binding has now been documented, few studies have quantitatively probed the importance of the phosphotyrosine binding site. However, several lines of evidence have suggested that electrostatic interactions are important in phosphotyrosine recognition. First, phosphorylation of the tyrosine is required for binding (*8*, *12*). Second, mutations that reduce the positive charge at the phosphotyrosine binding pocket have been shown to considerably decrease binding affinity (*13*). Finally, all structural studies have indicated

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that a large number of basic residues are located in the phosphotyrosine binding pocket (*14*). In a recent study (*15*), Singer and Forman-Kay used NMR spectroscopy to study the pH dependence of the interaction of the C-terminal SH2 domain of phospholipase C-γ (PLC-γ) with a high-affinity phosphopeptide and showed that the phosphotyrosine in the peptide undergoes a large change in pK_a between free and bound states. This observation indicates that, at least for the C-terminal PLC-γ SH2 domain, phosphopeptide binding is strongly linked to solution pH. However, it has remained unclear whether a large change in the phosphate pK_a accompanies binding for other SH2 domains. Furthermore, a thermodynamic description ($\Delta G°$, $\Delta H°$, $T\Delta S°$) of the linkage between peptide binding and protonation of the phosphate has yet to be determined.

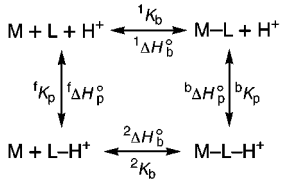
The role of ionizable groups in binding processes is often assessed by monitoring the association constant, *K*, of the interaction as a function of pH (*16*). However, this approach has limitations: *K* must be both experimentally accessible and also determined with a high degree of accuracy. Hence, in calorimetric studies of protein−ligand interactions, another method for studying proton linkage has been developed that involves determining the enthalpy of binding ($\Delta H°$) at different pHs and in buffers with different enthalpies of ionization ($\Delta H°_{ion}$) (*17*−*20*). Recent studies implementing this approach have provided detailed information about the linkage between binding and protonation energetics (*18*−*21*). However, in these studies, *K* was either not experimentally accessible (*20*) or only accessible over a very limited range (*18*, *21*), and hence no thorough comparison of the two methods of assessing proton linkages (i.e., *K* versus pH, and $\Delta H°$ versus pH and $\Delta H°_{ion}$) has yet been undertaken.

In the study presented here, the experimental accessibility to both *K* and $\Delta H°$ provided by isothermal titration calorimetry has been exploited to study the proton linkage involved in Src SH2 domain−phosphopeptide recognition. Two different series of experiments were designed such that one series focused on studying the pH dependence of *K*, while the other focused on determining $\Delta H°$ as a function of pH and $\Delta H°_{ion}$. It was found that each set of experiments provided similar, yet complementary, information regarding the pH linkage. In particular, a description of the energetic linkage between protonation of the phosphotyrosine and peptide binding was extracted. This study has provided for the first time a thermodynamic evaluation of the role of ionizable groups involved in SH2 domain recognition.

THEORY

Binding Linked to a Single Protonation Event. In this section, the expressions used to interpret the experimental data are presented. These expressions have been formulated based on the linkage theory developed by Wyman (*16*). The treatment of the pH linkage of calorimetric binding enthalpies has also been recently described by Murphy and co-workers (*19*, *20*).

Shown in eq 1 is a simple linkage scheme in which the binding of a ligand (L) to a macromolecule (M) is linked to protonation of the ligand. In this scheme, the subscripts p and b refer to the protonation and binding processes, respectively. The superscripts 1 and 2 refer to the binding of the deprotonated and protonated forms of the ligand, respectively, while the superscripts f and b refer to the protonation of the free and bound forms of the ligand.



(1)

Since the two processes of binding the ligand to the macromolecule and binding of a proton to the ligand together form a thermodynamic cycle, the association constants (K_b) of the macromolecule for the deprotonated (^1K_b) and protonated (^2K_b) ligand can be related to the proton binding constants (K_p) of the free (^fK_p) and bound (^bK_p) ligand by

$$(^1K_b)(^bK_p) = (^2K_b)(^fK_p) \tag{2}$$

Here, the proton binding constants, ^fK_p and ^bK_p, are related to the proton acid dissociation constants, ^fK_a and ^bK_a, by $^fK_p = 1/^fK_a$ and $^bK_p = 1/^bK_a$. Analogous to eq 2, the intrinsic enthalpies of protonation of the free ($^f\Delta H°_p$) and bound ($^b\Delta H°_p$) ligand are related to the intrinsic enthalpies of binding the deprotonated ($^1\Delta H°_b$) and protonated ($^2\Delta H°_b$) ligand to the macromolecule by

$$^1\Delta H°_b + {}^b\Delta H°_p = {}^2\Delta H°_b + {}^f\Delta H°_p \tag{3}$$

From simple linkage theory, it can be shown that the association constant (K_{obs}) as a function of pH is equal to the product of a reference equilibrium constant (here, ^1K_b) and the quotient of the subpartition functions for binding of protons to the bound and free forms of the ligand (Z_b and Z_f, respectively):

$$K_{obs} = {}^1K_b \frac{Z_b}{Z_f} \tag{4}$$

For the linkage of a single protonation event to binding, Z_b and Z_f are given by

$$Z_b = 1 + 10^{(pK_a)_b - pH} \tag{5}$$

and

$$Z_f = 1 + 10^{(pK_a)_f - pH} \tag{6}$$

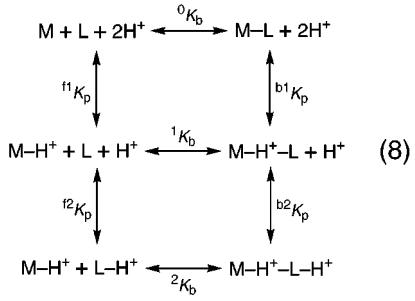
where $(pK_a)_b$ and $(pK_a)_f$ are related to ^bK_p and ^fK_p by $(pK_a)_b = \log {}^bK_p$ and $(pK_a)_f = \log {}^fK_p$. Hence, for a single proton linkage event, K_{obs} as a function of pH is given by

$$K_{obs} = {}^1K_b \frac{1 + 10^{(pK_a)_b - pH}}{1 + 10^{(pK_a)_f - pH}} \tag{7}$$

If experimental data are fit to eq 7, the parameters ^1K_b, $(pK_a)_b$, and $(pK_a)_f$ can be extracted.

[1] In general, the linkage of two protonation events to ligand binding would be schematically represented as a thermodynamic cube. However, if the pK_as of the two ionizable groups involved are widely separated, the thermodynamic scheme simplifies to the one depicted in eq 8.

Binding Linked to Two Protonation Events. Shown in eq 8 is a linkage scheme in which binding of a macromolecule to a ligand is dependent on two protonation events, one involving the macromolecule and the other involving the ligand.[1] The notation is as described above except that the superscripts f1 and f2 refer to the protonation of the free macromolecule and the free ligand, respectively, while the superscripts b1 and b2 refer to the protonation of the bound macromolecule and ligand, respectively. The superscript 0 refers to the binding of the deprotonated macromolecule and ligand.



(8)

In this case, the reference equilibrium constant has been designated to be ${}^1K_b$, the association constant of the deprotonated ligand to the protonated macromolecule. The association constant as a function of pH, K_{obs}, is again given by eq 4, but in this case, the subpartition functions (Z_b and Z_f) are given by

$$Z_b = 1 + 10^{(pK_a)_{b2}-pH} + 10^{pH-(pK_a)_{b1}} \quad (9)$$

and

$$Z_f = 1 + 10^{(pK_a)_{f2}-pH} + 10^{pH-(pK_a)_{f1}} \quad (10)$$

Hence, K_{obs} is given by

$$K_{obs} = {}^1K_b \frac{1 + 10^{(pK_a)_{b2}-pH} + 10^{pH-(pK_a)_{b1}}}{1 + 10^{(pK_a)_{f2}-pH} + 10^{pH-(pK_a)_{f1}}} \quad (11)$$

If experimental data are fit to eq 11, the parameters ${}^1K_b$, $(pK_a)_{b1}$, $(pK_a)_{f1}$, $(pK_a)_{b2}$, and $(pK_a)_{f2}$ can be determined.

pH Linkage of the Binding Enthalpy. A second method to access proton linkage is through the enthalpy of binding (*18*−*21*). The experimentally measured enthalpy (ΔH°_{obs}) of a calorimetry experiment at a given pH is determined by two values: the ionization enthalpy of the particular buffer employed (ΔH°_{ion}) and the enthalpy of the binding process corrected for buffer effects (ΔH°_b). These enthalpies are related to ΔH°_{obs} by

$$\Delta H^\circ_{obs} = \Delta H^\circ_b + (n)\Delta H^\circ_{ion} \quad (12)$$

where n is the number of protons absorbed upon binding of the ligand to the macromolecule. From eq 12, it is clear that ΔH°_b and n can be determined at a given pH from a series of experiments in which ΔH°_{obs} is measured in several buffers with different known values of ΔH°_{ion}.

If binding of a ligand to a macromolecule is linked to protonation of the ligand, both n and ΔH°_b will vary as a function of pH. n will be given simply by the following difference:

$$n = {}^bf - {}^ff \quad (13)$$

where ${}^bf$ and ${}^ff$ are the fractional saturation of protons at a given pH of the bound and free ligand, respectively. When ligand binding is linked to a single protonation event on the ligand (see eq 1), ${}^bf$ and ${}^ff$ can be easily expressed in terms of the pK_a of the ligand (in either its bound or free state) and the solution pH by

$${}^bf = \frac{10^{(pK_a)_b-pH}}{1 + 10^{(pK_a)_b-pH}} \quad (14)$$

and

$${}^ff = \frac{10^{(pK_a)_f-pH}}{1 + 10^{(pK_a)_f-pH}} \quad (15)$$

An expression for ΔH°_b, the enthalpy of the binding process, for the case of the single-proton linkage scheme depicted in eq 1 can be formulated in terms of the intrinsic enthalpies of binding and protonation (${}^1\Delta H^\circ_b$, ${}^2\Delta H^\circ_b$, ${}^b\Delta H^\circ_p$, and ${}^f\Delta H^\circ_p$) from eq 7 and the expression

$$\frac{\delta \ln K}{\delta(1/T)} = -\frac{\Delta H^\circ}{R} \quad (16)$$

where T is the temperature and R is the gas constant. This gives

$$\Delta H^\circ_b = {}^1\Delta H^\circ_b + ({}^bf){}^b\Delta H^\circ_p - ({}^ff){}^f\Delta H^\circ_p \quad (17)$$

which describes ΔH°_b in terms of the reference binding enthalpy of the deprotonated ligand (${}^1\Delta H^\circ_b$), the enthalpies of protonation of the bound (${}^b\Delta H^\circ_p$) and free ligand (${}^f\Delta H^\circ_p$), and the proton fractional saturation of the bound (${}^bf$) and free (${}^ff$) ligand. Finally, eqs 12 and 17 can be combined to give an expression of ΔH°_{obs} as a function of pH and ΔH°_{ion}:

$$\Delta H^\circ_{obs} = {}^1\Delta H^\circ_b + ({}^bf){}^b\Delta H^\circ_p - ({}^ff){}^f\Delta H^\circ_p + (n)\Delta H^\circ_{ion} \quad (18)$$

This expression describes each of the single-proton linkage parameters [$(pK_a)_f$, $(pK_a)_b$, ${}^1\Delta H^\circ_b$, ${}^b\Delta H^\circ_p$, ${}^f\Delta H^\circ_p$, and, through eq 3, ${}^2\Delta H^\circ_b$] in terms of the pH of the solution and the ΔH°_{ion} of the employed buffer. Experimental enthalpy data from calorimetry experiments performed at several different pH values and several different values of ΔH°_{ion} can be fit to eq 18 in order to determine the proton linkage parameters listed above. A similar, more complicated, expression (not shown here since it was not used in this study) can be derived to describe the linkage of more than a single protonation event to binding.

Hence, in theory, calorimetry provides two independent means to access the change in pK_a of an ionizable group involved in binding. However, to date, no thorough experimental comparison of these approaches has been undertaken.

MATERIALS AND METHODS

Protein Expression and Purification. The Src SH2 domain was expressed and purified as previously described (*5*, *11*). Protein concentration was assessed by using an extinction coefficient of $\epsilon_{280} = 14\,700\ M^{-1}\ cm^{-1}$ (*11*). The hamster

middle T antigen (hmT) phosphopeptide (sequence Ac-PQ-(pY)EEIPI-NH_2) was obtained from Quality Controlled Biochemicals (Hopkinton, MA). A phosphopeptide extinction coefficient of $\epsilon = 538\ M^{-1}\ cm^{-1}$ at 268 nm and pH 6.0 was obtained for the hmT peptide by mass and was confirmed by quantitative amino acid analysis. Extinction coefficients for the hmT peptide at values of pH other than pH 6.0 were determined from the extinction coefficient at pH 6.0 and the pH titration curve described below.

pH Titrations. To determine the pK_a of the peptide's phosphotyrosine (pTyr) by pH titration, 1 mL of a 1.0 mM solution of hmT peptide solution was typically titrated with ~2 μL aliquots of 1.33 M acetic acid. The absorbance at 268 nm of the hmT peptide at each pH was recorded on a Varian dual beam spectrophotometer. pH titrations in the triple buffer (described below) were typically performed from pH 8.0 to 4.0, while titrations in the single MES buffer were performed over a more limited range. To determine the apparent pK_a of the pTyr, the absorbance data were fit to the following expression:

$$\mathrm{Abs} = \frac{(A1 - A2)}{1 + 10^{\mathrm{p}K_a - \mathrm{pH}}} + A2 \qquad (19)$$

where the absorbance (Abs) is given as a function of pH, and the fitted parameters are the apparent pK_a of the ionizable group, as well as the absorbance of the fully deprotonated ($A1$) and fully protonated ($A2$) form.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed with an Omega titration microcalorimeter (Microcal Inc., Northhampton, MA) (*22*). The typical concentrations of the Src SH2 domain and hmT peptide employed in ITC experiments, as well as the steps to prepare both the protein and peptide, have been previously described (*11*). All experiments were performed at 25 °C. The buffer used in the first series of experiments aimed at determining K_{obs} was a triple buffer consisting of 25 mM sodium acetate, 25 mM MES, 50 mM Tris-HCl, 150 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA (*23*). The buffer conditions in the second series of experiments aimed at determining $\Delta H°_{obs}$ as a function of pH and $\Delta H°_{ion}$ were 20 mM buffer (sodium acetate, sodium cacodylate, Aces, Bistris, Hepes, MES, Tricine, or Tris-HCl), 50 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA. The $\Delta H°_{ion}$ of the employed buffers was taken from literature values (*17*, *24*−*26*). The Src SH2 domain and hmT peptide were dialyzed into the appropriate calorimetry buffer for 24−48 h prior to ITC experiments.

Analysis of ITC Data. The raw data from the calorimetry experiments were collected and integrated with the ORIGIN software (*22*). Experiments in which the hmT peptide was injected into buffer alone demonstrated that the heat of dilution of the hmT peptide was very similar to the evolved heat observed at the end of a titration of the protein with peptide; therefore, the baseline of the titrations of the Src SH2 domain with the hmT peptide could typically be well estimated from the last injections of a titration. The integrated data from all experiments fit well to a single-site binding model, with the stoichiometry of binding typically falling between 0.9 and 1.1. The c value, the product of K_{obs} and the Src SH2 domain concentration, varied from 7 to 400 for all experiments and therefore was in a range for which accurate values of K_{obs} can be obtained (*22*).

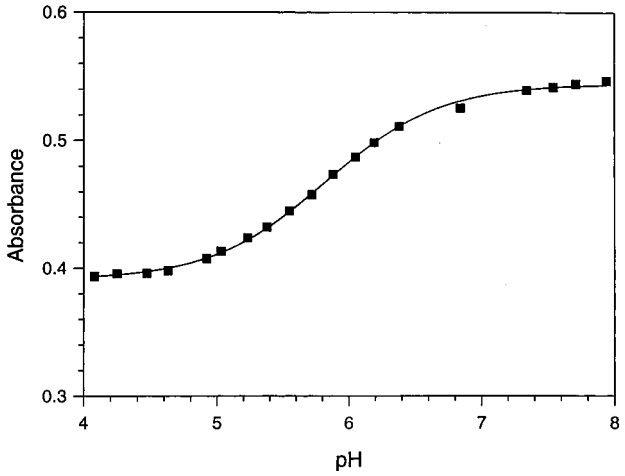


FIGURE 1: pH titration of the hmT phosphopeptide. Plotted is the peptide absorbance (268 nm) versus pH. The titration shown was performed in a buffer of 25 mM sodium acetate, 25 mM MES, 50 mM Tris-HCl, 150 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA, at 25 °C.

The thermodynamic parameters derived from the calorimetry experiments were fit to the various proton linkage models with the program Scientist (Micromath, Salt Lake City, UT). For the log K_{obs} versus pH fitting, 27 independent values of K_{obs} were used to determine the five variable parameters. For the fitting of $\Delta H°_{obs}$ as a function of pH and $\Delta H°_{ion}$, 50 independent experiments were used to determine the 5 variable parameters. The reported uncertainties in all parameters represent 67% confidence intervals.

RESULTS

Isothermal titration microcalorimetry (ITC) has been employed to characterize the binding of the Src SH2 domain to a peptide based on the hamster middle T antigen (hmT). The use of ITC permits a direct determination of the heat (or enthalpy, $\Delta H°$) evolved from a binding process in addition to the binding affinity (or association constant, K). From these parameters, the Gibbs free energy ($\Delta G°$) and the entropy of binding ($\Delta S°$) can be directly calculated, while the heat capacity change ($\Delta Cp°$) can be obtained from the temperature dependence of the binding process. Furthermore, the number of protons exchanged upon binding, n, can be determined from eq 12 by measuring $\Delta H°_{obs}$ from calorimetry experiments performed in different buffers with different values of $\Delta H°_{ion}$. Recently, this information has been obtained for the interaction of the Src SH2 domain with the hmT peptide (*11*). It was revealed that at pH 6.0 a net release of protons accompanied binding, indicating the existence of a linkage between hmT peptide binding and protonation of an ionizable group. In this study, the basis of this proton linkage has been investigated.

Determination of the pK_a of pTyr. The absorbance at 268 nm of the hmT phosphopeptide was observed to change with the pH of the solution, indicating that the ionization state of the pTyr, the only absorbing group at this wavelength, is affected by pH. Therefore absorption spectroscopy was used to determine the pK_a of the pTyr by acid−base titration of the hmT peptide (Figure 1). Fitting the data to eq 19 indicated that the apparent pK_a of the pTyr phosphate group

Table 1: Thermodynamic Binding Parameters of the hmT Peptide in the Triple Buffer

pH	no. of expts	K_{obs} (M^{-1})	$\Delta G°_{obs}$[a] (kcal mol^{-1})	$\Delta H°_{obs}$[a] (kcal mol^{-1})
5.0	3	$1.7 (\pm 0.4) \times 10^5$	−7.1	−6.5
5.5	3	$4.6 (\pm 0.6) \times 10^5$	−7.7	−6.9
6.0	3	$8.8 (\pm 1.8) \times 10^5$	−8.1	−7.4
6.5	3	$1.8 (\pm 0.7) \times 10^6$	−8.4	−7.5
7.0	3	$1.8 (\pm 0.5) \times 10^6$	−8.5	−7.3
7.5	3	$2.4 (\pm 1.0) \times 10^6$	−8.7	−6.9
8.0	3	$1.6 (\pm 0.5) \times 10^6$	−8.4	−6.0
8.5	3	$1.6 (\pm 0.2) \times 10^6$	−8.4	−5.8
9.0	3	$1.2 (\pm 0.9) \times 10^6$	−8.3	−5.8

[a] Standard deviations of the three multiple experiments were typically ±0.2 and ±0.5 for $\Delta G°_{obs}$ and $\Delta H°_{obs}$, respectively. The buffer conditions in these experiments were 25 mM sodium acetate, 25 mM MES, 50 mM Tris-HCl, 150 mM NaCl, 1 mM β−mercaptoethanol, and 1 mM EDTA, 25 °C.

was equal to 6.1 ± 0.1. This value is very similar to the pK_as determined for other pTyr-containing peptides (*15*, *27*) and was independent of the buffer conditions employed in the ITC experiments described below. This value is also close to the apparent pK_a of the free amino acid pTyr, which was determined to be 5.8 ± 0.1 (data not shown).

Measurement of K_{obs} as a Function of pH. To probe the linkage between the ionization of the hmT peptide phosphate and hmT peptide binding to the Src SH2 domain, a series of ITC experiments was designed to examine the hmT peptide association constant, K_{obs}, as a function of pH. ITC experiments were performed in triplicate from pH 5.0 to 9.0 in 0.5 pH unit intervals. For these experiments, a triple buffer was used that allowed for constant buffering conditions and ionic strength throughout the investigated pH range (*23*). This buffer also contained a higher salt concentration than previously employed (*11*) in order to lower the affinity to a range that was optimal for accurate determination of K_{obs} by ITC. All titrations were exothermic, and the data from each experiment fit well to a single-site binding model. The data derived from these calorimetric titrations are shown in Table 1. K_{obs} increases ~20-fold from pH 5.0 to 7.5 but still could be accurately measured at all pH values. The experimentally measured $\Delta H°_{obs}$ is also shown in Table 1; however, $\Delta H°_{obs}$ is difficult to interpret in terms of proton linkage due to the different enthalpic effects that arise from the exchange of protons with the various buffer components.

Figure 2 shows the plot of log K_{obs} versus pH. The curve has a maximum near pH 7.5 and decreases above and below this value, which indicates that at least two ionization events are linked to binding. However, the decreasing trend in log K_{obs} is more dramatic at low pH than high pH, implying a stronger proton linkage at low pH.

These data were fit to various models of proton linkage involving the change in pK_a of ionizable groups upon binding. A single-proton linkage model (eq 7) could not adequately describe the data over the entire range of pH, although it described the data well over the more limited range of pH 5.0−7.5. Hence the data over the entire range of measurements were fit to a two-proton linkage model (eq 11). This two-proton linkage model describes the data well (Figure 2), and the best-fit parameters for this model are given in Table 2. The value for $(pK_a)_{f2}$, the free pK_a of the group that ionizes at low pH, was determined to be 6.2, in

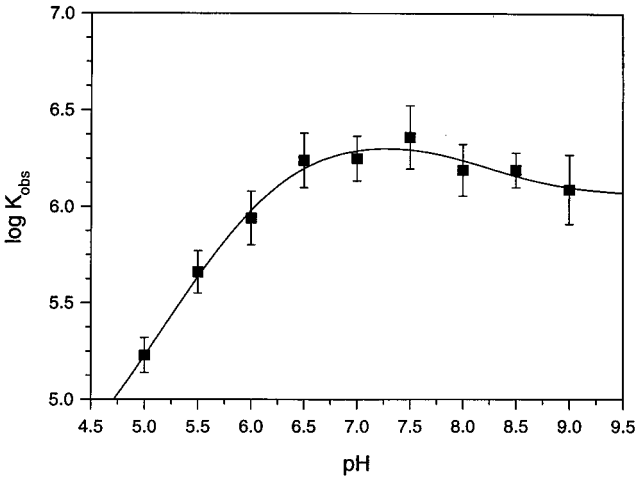


FIGURE 2: Association constant (K_{obs}) of the hmT peptide as a function of pH. For these experiments, a triple buffer of 25 mM sodium acetate, 25 mM MES, 50 mM Tris-HCl, 150 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA was employed. Error bars represent the standard deviation of three independent experiments. The solid line is the best fit to a two-proton linkage model as described in the text.

Table 2: Best-Fit Parameters for the Two-Proton Linkage Model Applied to the log K_{obs} versus pH Data[a]

^1K_b (M^{-1})	$2.2 (\pm 0.4) \times 10^6$
$(pK_a)_{f1}$	8.2 ± 0.7
$(pK_a)_{b1}$	8.5 ± 0.7
$(pK_a)_{f2}$	6.2 ± 0.2
$(pK_a)_{b2}$	4.4 ± 0.7
$n(5.2)^b$	−0.77
$n(6.0)^b$	−0.58
$n(7.0)^b$	−0.13
$n(8.0)^b$	0.15

[a] Best-fit parameters are determined from nonlinear least-squares fitting to eq 11 using the calorimetric data depicted in Table 1. Uncertainties represent 67% confidence intervals. [b] Determined from the best fit to the two-proton model from $\delta \log K_{obs}/\delta$ pH.

good agreement with the known free pK_a of the pTyr in the peptide, which is 6.1 ± 0.1. The corresponding bound pK_a value of this group, $(pK_a)_{b2}$, is 4.4, showing that a significant downward pK_a shift occurs upon binding. The free and bound pK_a values for the group ionized at high pH [$(pK_a)_{f1}$ and $(pK_a)_{b1}$] are 8.2 and 8.5, respectively, which indicates that an upward shift in pK_a occurs upon binding for this group. However, it should be noted that $(pK_a)_{f1}$, $(pK_a)_{b1}$, and $(pK_a)_{b2}$ are not determined with a high degree of certainty, due to the small decrease in K_{obs} at high pH and, at least partially, to the intrinsic uncertainty in the data. The number of protons exchanged upon peptide binding at each pH, *n*, which is determined from the slope of the log K_{obs} versus pH curve, is also reported in Table 2. The greatest release of protons occurs at pH 5.0, reflecting the dramatic decrease in K_{obs} at low pH.

Measurement of $\Delta H°_{obs}$ as a Function of pH and $\Delta H°_{ion}$. To further dissect the pH linkage of the Src SH2 domain−hmT peptide interaction, a second series of ITC experiments was performed. These experiments were designed to use the enthalpy of binding, $\Delta H°_{obs}$, as a second independent probe of the protonation energetics (see Theory and refs *19* and *20*). Calorimetry experiments were performed under the standard salt conditions (*11*) and at pH values of 4.5, 5.2,

Table 3: Calorimetric Data for Src SH2 Domain Binding to the hmT Peptide at Different Values of pH

pH	buffer	$\Delta H°_{ion}$ (kcal/mol)	no. of expts	$\Delta G°_{obs}$[a] (kcal/mol)	$\Delta H°_{obs}$[a] (kcal/mol)	$\Delta H°_{fit}$[b] (kcal/mol)
4.5	acetate	0.00	3	−7.5	−6.4	−6.5
5.2	cacodylate	−0.56	2	−7.7	−5.8	−5.9
5.2	acetate	0.00	2	−8.4	−6.3	−6.3
5.2	MES	3.73	4	−8.7	−9.5	−9.1
5.2	Bistris	6.75	2	−8.1	−11.0	−11.4
6.0	cacodylate	−0.56	5	−8.8	−6.2	−6.2
6.0	MES	3.73	3	−9.1	−8.4	−8.5
6.0	Bistris	6.75	2	−9.2	−10.4	−10.2
6.0	ACES	7.47	5	−9.4	−10.3	−10.6
7.0	cacodylate	−0.56	2	−9.0	−6.2	−6.8
7.0	MES	3.73	2	−9.6	−7.3	−7.3
7.0	Hepes	5.00	4	−9.6	−7.7	−7.4
7.0	Bistris	6.75	1	−9.5	−8.0	−7.6
7.0	ACES	7.47	4	−9.5	−7.7	−7.7
8.0	Hepes	5.00	4	−9.1	−7.0	nd
8.0	Tricine	7.76	3	−9.2	−6.5	nd
8.0	Tris-HCl	11.51	4	−9.4	−5.9	nd

[a] Standard deviations of multiple experiments were typically ±0.3 and ±0.4 kcal/mol for $\Delta G°_{obs}$ and $\Delta H°_{obs}$, respectively. [b] Derived from the best-fit parameters for the single-proton linkage model (eq 18). The buffer conditions in these experiments were 20 mM buffer, 50 mM NaCl, 1 mM β−mercaptoethanol, and 1 mM EDTA, 25 °C. nd, not determined.

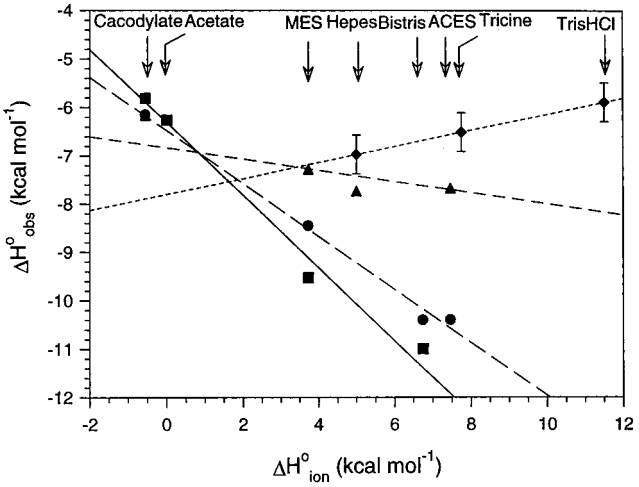


FIGURE 3: Enthalpies of binding for the Src SH2 domain binding to the hmT peptide at pH 5.2 (■), pH 6.0 (●), pH 7.0 (▲), and pH 8.0 (◆) as a function of the $\Delta H°_{ion}$ of the buffer. The buffer conditions in these experiments were 20 mM buffer, 50 mM NaCl, 1 mM β-mercaptoethanol, and 1 mM EDTA. The typical standard deviation among independent experiments is depicted only for the pH 8.0 data, but is typical for experiments at all pH values. The predicted $\Delta H°$ values at pH 5.2 (solid line), pH 6.0 (long dashed line), and pH 7.0 (short dashed line) as a function of $\Delta H°_{ion}$, derived from the global fit to a single proton linkage model, are also depicted. The dotted line depicts the linear best fit to the pH 8.0 $\Delta H°_{obs}$ data. Arrows indicate the $\Delta H°_{ion}$ values for the buffers employed.

6.0, 7.0, and 8.0 in several different single-component buffers with different $\Delta H°_{ion}$. Due to an insufficient spread in the known $\Delta H°_{ion}$ of buffers at high and low pH, experiments at pH higher than 8.0 were not attempted and only a single buffer (sodium acetate) was used at pH 4.5.

A potential complication of studying the pH dependence of the binding process in numerous buffer systems is that the different buffer components may interact with the protein or peptide in such a way as to provide a large difference in the intrinsic thermodynamics of peptide binding between different buffers. However, in the present study, this was not the case since the peptide $\Delta G°$ was found to be relatively independent of the employed buffer at a given pH (with the possible exception of the experiments at pH 5.2 and 7.0 in sodium cacodylate; see Table 3).

As shown in Table 3, the experimental parameter of interest from these experiments, $\Delta H°_{obs}$, is dependent on both the pH of the solution and the employed buffer. In fact, the measured enthalpy varies widely from $\Delta H°_{obs} = -5.8$ kcal mol^{-1} in sodium cacodylate, pH 5.2, to $\Delta H°_{obs} = -11.0$ kcal mol^{-1} in Bistris, pH 5.2 (Table 3). The slopes of plots of $\Delta H°_{obs}$ versus $\Delta H°_{ion}$, which give the number of protons exchanged upon binding, n, are constant at each pH (Figure 3). However, n increases with increasing pH [$n(5.2) = -0.74$, $n(6.0) = -0.48$, $n(7.0) = -0.19$, and $n(8.0) = 0.17$], indicating that the greatest amount of proton release upon peptide binding occurs at pH 5.2, while hmT peptide binding occurs with an uptake of 0.17 proton at pH 8.0.

Since the log K_{obs} vs pH data within the restricted pH range of 5.0−7.5 were well described by a single protonation event, the subset of $\Delta H°_{obs}$ data from pH 4.5 to 7.0 was fit to a single-proton linkage model (see eq 18). This model provided a good description of the data. As shown in Table 3, the nonlinear least-squares best fit to the model ($\Delta H°_{fit}$) was able to predict the enthalpy at each value of pH and $\Delta H°_{ion}$ within ±0.5 kcal mol^{-1}. The straight lines in Figure 3, which represent the best fit to the single-proton linkage model, further demonstrate the validity of this model. The data collected at pH 8.0 were not sufficient to fully characterize the ionizable group linked to binding at high pH, but the increasing trend in the $\Delta H°_{obs}$ vs $\Delta H°_{ion}$ plot at pH 8.0 is consistent with the triple buffer experiments, which showed that an uptake of protons occurred upon formation of the SH2 domain−phosphopeptide complex at pH 8.0 (Table 2).

The best-fit parameters of the single-proton linkage model, the free and bound pK_as of the ionizable group [$(pK_a)_f$ and $(pK_a)_b$], the enthalpies of binding of the two forms of the peptide ($^1\Delta H°_b$ and $^2\Delta H°_b$), and the ionization enthalpies of the group involved ($^b\Delta H°_p$ and $^f\Delta H°_p$), are reported in Table 4. The free pK_a of the ionizable group linked to binding [$(pK_a)_f$] is equal to 6.1 ± 0.1, in good agreement with the known pK_a of pTyr, while the pK_a in the bound form [$(pK_a)_b$] is 4.4 ± 0.3. Both values are nearly identical to those derived from the analysis of log K_{obs} versus pH in the triple buffer experiment (Table 2). However, the value of pK_{ab} obtained here is more precisely determined than that obtained in the triple buffer experiments [see $(pK_a)_{b2}$ in Table 2]. The value of the protonation enthalpy of the unbound peptide phosphate ($^f\Delta H°_p = -0.8 \pm 0.7$ kcal mol^{-1}) is also in good agreement with the known enthalpy of protonation of the phosphate ion [−1.1 kcal mol^{-1} (*24*)].

Determination of the Thermodynamic Linkage between Protonation and Binding at Low pH. The preceding experiments have provided a thermodynamic description ($\Delta G°$, $\Delta H°$, $\Delta S°$) of the linkage between ionization of the pTyr's phosphate and peptide binding (Table 4). Since the pK_a of the pTyr was shifted downward by 1.7 pH units upon binding, the PO_3^{2-} form of the hmT peptide binds the Src SH2 domain 1.7 log units more favorably than the PO_3H^{1-}

Table 4: hmT Peptide Binding and Protonation Energetics[a]

$\log {}^1K_b$[b]	7.0 ± 0.2
$\log {}^2K_b$[c]	5.3 ± 0.4
$(pK_a)_f$[d]	6.1 ± 0.1
$(pK_a)_b$[d]	4.4 ± 0.3
${}^1\Delta G°_b$[e] (kcal mol^{-1})	-9.5 ± 0.3
${}^2\Delta G°_b$[e] (kcal mol^{-1})	-7.2 ± 0.5
${}^f\Delta G°_p$[e] (kcal mol^{-1})	-8.3 ± 0.1
${}^b\Delta G°_p$[e] (kcal mol^{-1})	-6.0 ± 0.4
${}^1\Delta H°_b$[d] (kcal mol^{-1})	-6.9 ± 0.3
${}^2\Delta H°_b$[d] (kcal mol^{-1})	-6.9 ± 1.1
${}^f\Delta H°_p$[d] (kcal mol^{-1})	-0.8 ± 0.7
${}^b\Delta H°_p$[d] (kcal mol^{-1})	-0.8 ± 1.3
$T{}^1\Delta S°_b$[f] (kcal mol^{-1})	2.6 ± 0.4
$T{}^2\Delta S°_b$[f] (kcal mol^{-1})	0.3 ± 1.2
$T{}^f\Delta S°_p$[f] (kcal mol^{-1})	7.5 ± 0.7
$T{}^b\Delta S°_p$[f] (kcal mol^{-1})	5.2 ± 1.4

[a] Refer to eq 1 to identify the components of the linkage cycle. [b] Taken from experiments in ACES buffer, pH 7.0. [c] Calculated from eq 2. [d] Determined from eq 18. [e] Calculated from $\Delta G° = -RT \ln K$. [f] Calculated at 25 °C from $T\Delta S° = \Delta H° - \Delta G°$. Uncertainties either represent 67% confidence intervals from the best-fit parameters of the one proton linkage model or otherwise are calculated with standard error propagation.

form of the peptide; this amount is equivalent to a 2.3 kcal mol^{-1} more favorable $\Delta G°$ of binding, which is ~25% of the total binding free energy of the hmT peptide. Furthermore, since the enthalpies of binding of the two different forms of the hmT peptide (${}^1\Delta H°_b$ and ${}^2\Delta H°_b$) are indistinguishable, so are the enthalpies of protonation of the peptide phosphate in the free and bound states (${}^f\Delta H°_p$ and ${}^b\Delta H°_p$). Hence, the entropy must be the parameter that distinguishes both the binding of the two different forms of the peptide and the ionization of the free and bound forms of the peptide phosphate (Table 4).

DISCUSSION

The two parameters that can be obtained from an ITC experiment, the association constant K and the enthalpy $\Delta H°$, have each been used independently to investigate the linkage between pH and Src SH2 domain binding to the high-affinity hmT phosphopeptide. The two approaches provided self-consistent and complementary information regarding the recognition process.

The experiments in this study are well described by a model in which two ionizable groups are linked to binding: one group (the pTyr) below pH 7.0 and another above pH 7.5. pH titration studies using NMR on the related C-terminal SH2 domain of PLCγ support the assignment of the low-pH protonation energetics solely to the pTyr (*15*). These studies demonstrated that a change in the pTyr pK_a from 6.1 to 4.0 occurred upon peptide binding to the PLCγ SH2 domain, which is similar to the pK_a shift measured here for the Src SH2 domain. Furthermore, in this previous study, no other residue studied besides the pTyr was shown to undergo a significant change in pK_a upon peptide binding; His βD4 (see notation in refs *5* and *7*), which is located near the pTyr binding pocket, had a pK_a less than 4.0 in both the peptide-free and peptide-bound forms of the PLCγ SH2 domain (*15*), likely due to the highly positively charged environment of the pTyr binding site. Since the environment of His βD4 is similar in the Src SH2 domain (Figure 4), this residue likely does not contribute to the protonation energetics. Two other ionizable groups, the Glu residues at positions +1 and +2 of the hmT peptide, could have also contributed to the observed pH linkage. However, the free amino acid glutamate in solution typically has a pK_a near 4.3, which is below the pH range employed in this study. Furthermore, the Glu residues in the hmT peptide will likely undergo a decrease in pK_a upon binding since these residues are involved in ionic interactions with positively charged residues in the SH2 domain (*6*, *28*). Hence, the +1 and +2 Glu residues are also unlikely to significantly contribute to the experimentally measured proton linkage.

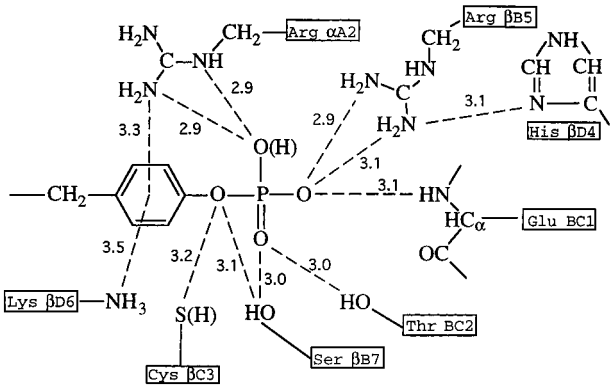


FIGURE 4: Schematic diagram of the pTyr binding pocket (*6*). Dashed lines indicate hydrogen-bonding interactions and interactions with the tyrosine ring. Distances between non-hydrogen atoms (and with the center of the ring) are shown. Ionizable protons within the range of pH studied are indicated in parentheses.

The magnitude of decrease in the pTyr pK_a upon binding (1.7 pK_a units) is consistent with other studies that have examined the binding of a phosphate moiety to positively charged sites on other proteins. For example, the binding of the 2′-phosphate of NADP$^+$ either to dihydrofolate reductase or to isocitrate dehydrogenase has been shown to reduce the free pK_a of the phosphate by at least 2 pK_a units (*29*, *30*).

There is insufficient evidence to definitively assign the proton linkage above pH 7.5 to a specific residue. One ionizable residue that could be responsible for the determined energetics is Cys βC3, which participates in forming the pTyr binding pocket of the Src SH2 domain (Figure 4). The amino acid cysteine has a pK_a value of 8.3, but cysteine residues in proteins may more typically have pK_a values near 9.0 (*31*); either value would be consistent with the data obtained from the pH dependence of the association constant (Table 2). Since the βC3 position is a Ser in all other SH2 domains of the Src family (Lck, Fyn, etc.), it would be interesting to examine whether other members of this family of SH2 domains show a similar pH dependence to binding above pH 7.5.

Our studies have found that the PO_3^{2-} form of the hmT peptide binds more favorably than the PO_3H^{1-} form by 2.3 kcal mol^{-1}. This result demonstrates that electrostatic interactions within the pTyr binding pocket play a critical role in promoting high-affinity binding. In fact, the loss of a single negative charge from the phosphate causes nearly as great a loss in binding free energy as substituting Ala at all three peptide positions C-terminal to the pTyr (Bradshaw and Waksman, unpublished results). The origin of the large contribution of the PO_3^{2-} form to binding may be related to

the presence of numerous unpaired positive charges within close proximity in the pTyr binding site, one of which, Arg βB5, is partially buried. The two negative charges associated with the PO_3^{2-} form of the phosphate are apparently required to fully satisfy the ionic bonding potential of these charges as well as to alleviate their destabilizing effect on the free state of the SH2 domain.

Table 4 shows that the shift in the pTyr pK_a upon binding is due entirely to entropic effects, since the enthalpy of binding is equivalent for the PO_3^{2-} and PO_3H^{1-} forms of the peptide. The more favorable $\Delta S°_b$ for binding of the PO_3^{2-} form of the peptide compared to the PO_3H^{1-} form is consistent with studies which have shown that formation of ionic bonds in protein−protein interactions is often entropically favorable (*32*). However, the identical values of ${}^1\Delta H°_b$ and ${}^2\Delta H°_b$ (and ${}^b\Delta H°_p$ and ${}^f\Delta H°_p$) determined here are somewhat puzzling. For example, a significantly more favorable $\Delta H°_p$ was determined for the binding of a proton to free elastase than for proton binding to a complex of elastase with ovomucoid third domain (*20*); this was attributed to an "enthalpic penalty" of stripping the water from the proton upon binding within an enclosed cavity of the ovomucoid third domain−elastase complex. However, in our study, since ${}^b\Delta H°_p$ and ${}^f\Delta H°_p$ (and hence ${}^1\Delta H°_b$ and ${}^2\Delta H°_b$) are identical, this effect must be small or otherwise compensated by some other enthalpic contribution.

The critical nature of the ionic bonds involving the PO_3^{2-} form of the peptide ligand has significant implications for the design of SH2 domain binding inhibitors. Due to the need for such inhibitors to penetrate the cell membrane, considerable efforts have been made designing compounds with reduced charge (*9*, *33*). However, these attempts have failed to produce effective drugs. It is now clear from the study presented here why these efforts may have not succeeded: the loss of one charge on the phosphate is greatly detrimental to binding. The design of inhibitors for the Src family of SH2 domains is further complicated by the fact that these SH2 domains bind with highest affinity to phosphopeptides with four net negative charges (*8*, *34*). Therefore, it appears that the central role played by ionizable groups in Src SH2 domain binding will provide a challenge to the design of small, uncharged inhibitors of Src SH2 domain recognition.

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