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Experimental and Calculated Shift in pK_a upon Binding of Phosphotyrosine Peptide to the SH2 Domain of p56^{lck}

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Abstract—The pH dependence of the affinity of a 11-mer phosphotyrosine (pY) peptide (EPQpYEEIPIYL-NH2) for the SH2 domain of the tyrosine kinase p56^{lck} was investigated with surface plasmon resonance (SPR). From SPR competition experiments the affinity in solution was obtained. The pH dependence of the affinity in solution can be well described by a proton linkage model with a single pK_a shift upon binding, from 6.1 to 4.7. This shift is ascribed to the transition from the −2 to the −1 ionisation state of the tyrosine phosphate group. Based on the X-ray structure for the complex with Lck SH2, a pK_a value of 5.3 for the bound pY peptide was computed, modelling the solvated protein as a system of point charges in a continuum. With the phosphate in the −2 state the binding energy is 1.8 kcal/mol more favourable than for the −1 state, corresponding to a 20-fold higher affinity. A proper charge is relevant in the design of potential therapeutic Lck SH2 ligands with mimics for the metabolically unstable tyrosine phosphate group.

Introduction

The binding of ligands to proteins generally shows some degree of pH dependence. This phenomenon of proton-linkage reflects the coupling of ligand binding and proton binding equilibria. Proton linkage effects can be ascribed to structural features of the protein binding pocket and the ligand, through the assignment of microscopic pK_a values to specific functional groups. This information is relevant for structure based ligand design, where the ionisation state should be chosen for optimal affinity.

We determined the pH dependence of the binding of a phosphotyrosine (pY) peptide to the SH2 domain of the tyrosine kinase p56^{lck} (Lck SH2). SH2 domains are protein modules of approximately 100 amino acids that recognise specific phosphotyrosine-containing sequences. They occur in numerous proteins involved in signal transduction.[1,2] There is much interest in the design of ligands for SH2 domains, as such ligands may be potential drugs for cancer, allergy, asthma and inflammatory diseases.[3–5] As the tyrosine phosphate group is prone to enzymatic hydrolysis, considerable effort has been directed to finding mimics for phosphotyrosine (e.g., see refs 6–8). The ionisation state of these phosphate mimics should be optimal for affinity and agree with the high affinity binding state of phosphotyrosine ligands.

pH dependence of binding of phosphotyrosine peptides to SH2 domains has been studied by Singer and Forman-Kay[9] for the phospholipase C-γ_1 C-terminal SH2 domain by NMR, they report a shift in the pK_a for the phosphotyrosine group from 6.1 to 4.0. Bradshaw and Waksman[10] reported free and bound pK_as of 6.2 and 4.4, respectively, for the Src SH2 domain investigated by isothermal titration calorimetry (ITC). Additionally, they found another pK_a shift with free and bound pK_as of 8.2 and 8.5, respectively, which was tentatively assigned to a cysteine in the phosphotyrosine binding pocket.[10] Here we present the pH dependence of the affinity for Lck SH2 of a phosphotyrosine 11-mer peptide derived from the hamster middle T antigen (hmT). The Lck SH2 domain is rather similar to Src SH2, recognising the same consensus sequence, however, some differences exist in the phosphotyrosine binding pocket, for example, the Lck SH2 domain lacks the above mentioned cysteine residue.

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It is a challenge to compute reliable pK_a values. Effective pK_as of ionisable groups in proteins can be derived using finite-difference Poisson–Boltzmann calculations, modelling the solvated protein as a system of point charges in a continuum, with separate dielectric constants for protein and solvent.[11,12] As the crystal structure of Lck SH2 in complex with the hmT 11-mer is available, we explored such pK_a calculations in the bound state.

Results

The pK_a of the free hmT 11-mer peptide (EPQpYEEIPIYL-NH_2) in the triple buffer used in the affinity experiments, was assayed by absorption measurements at 268 nm (Fig. 1). As pY and Y are the only absorbing residues in the peptide at this wavelength, the change in absorption with pH can be ascribed to the tyrosine phosphate group. The obtained pK_a value of 6.05 ± 0.05 is close to that for the transition of the -1 to -2 state of free phosphotyrosine and various pY-peptides.[9,10]

The affinity of the hmT peptide for Lck SH2, as a function of pH is obtained from SPR experiments. With SPR one measures the change in SPR angle upon binding of an analyte, the SH2 domain in this case, to an immobilised ligand on the SPR sensor chip. From SPR experiments in the presence of various concentrations of competing pY peptide, a binding constant for the interaction in solution is obtained (K_S, see Experimental). Affinity constants obtained in this way compared well with the solution affinity determined with a fluoresence assay using a labelled pY-peptide (results not shown). Our analysis of the SPR data uses the SPR signal at equilibrium (R_{eq}), which is generally reached within 10 min.[13] In Figure 2, typical inhibition curves at various pH values are shown. To obtain K_S for the interaction in solution, the binding constant for the interaction at the SPR sensor chip (K_C), as obtained from SPR experiments without competing peptide, must first be known (see Experimental, eq 4). This value is obtained from non-linear fit of the binding isotherm (Fig. 3).

In Fig. 4 the affinity for the interaction at the SPR sensor surface and in solution is shown as function of pH (K_{obs}). The affinity at the sensor surface is higher than that in solution. An avidity effect due to formation of dimers between the GST parts of the SH2 fusion protein at the sensor surface is probably responsible for this.[14] The interaction at the chip shows some decrease in affinity at pH >7, while the affinity in solution is not affected. Figure 4 shows that the data for the interaction in solution can be well described by a one-proton linkage model (eq 8). For the interaction at the sensor surface the more complicated pH dependence can be described by a two-proton linkage model (eq 9). The parameters obtained from fits according to the proton linkage models are shown in Table 1. The pK_a shifts in the pH region from 6 to 4 can be assigned to changes in the ionisation state of the tyrosine phosphate group, in particular to the change from the -1 ionisation state to the -2 state.[9,10] The hmT peptide also contains ionisable groups from glutamate residues but the pK_a value of these groups is lower than the pH range employed in this study. In the fits according to the proton-linkage models, the experimental pK_a value of 6.05 for the free hmT peptide (pK^f) was used. Fits with pK^f floating, yield similar results with pK^f values of 5.9 ± 0.1 and 6.1 ± 0.1 from binding at the sensor surface, and in solution, respectively. The second pK_a shift from 7.4 to 8.0 for binding at the sensor surface is tentatively ascribed to GST dimerisation. It has been reported that several salt bridges are involved in GST dimerisation.[14]

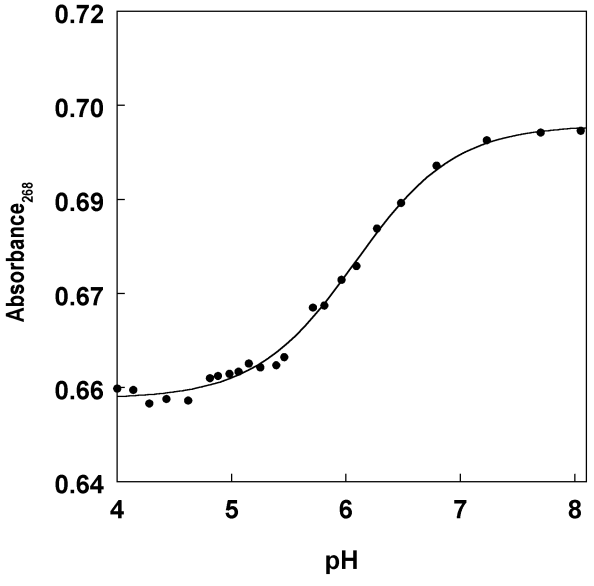


Figure 1. pH titration of hmT tyrosine phosphate 11-mer peptide in triple buffer at 25 °C. Initial peptide concentration 0.47 mM. The line is the fit according to eq 10. The pK_a value from three experiments is 6.05 ± 0.05.

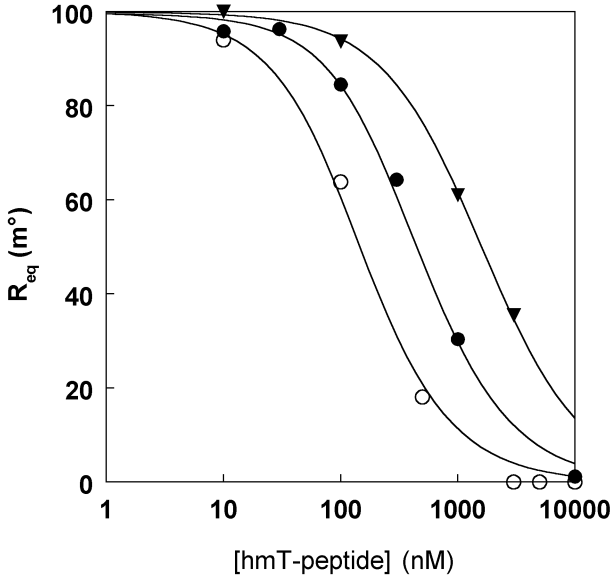


Figure 2. SPR competition experiments. The SPR signal at equilibrium (R_{eq}) in millidegree (m°) for binding of Lck SH2 to immobilised hmT peptide in the presence of various concentrations of competing hmT peptide. Experiments in triple buffer at 25 °C: pH 9.0 ○; pH 6.8 ●; pH 5.0 ▼. The lines are the fits according to (eq 4), in which (eq 5) has been substituted. From the fits the affinity in solution is obtained (K_S). Concentration Lck SH2 is 50 nM, except for pH 9.0 where it is 30 nM. N.B. For ease of comparison the curves have been normalised to R_{eq} in the absence of competing peptide of 100 m°.

At pH <7 the curves for K_S and K_C are parallel and the pK_a shift of the phosphotyrosine for binding in solution is identical to that for binding at the sensor (Table 1). This indicates that the binding mode around the immobilised phosphotyrosine moiety is identical to that in solution. Such a conclusion is in line with results from a thermodynamic study of the hmT peptide binding to Lck SH2, where we found that the enthalpy and heat capacity changes for binding in solution are similar to those at the sensor surface (de Mol et al., manuscript in preparation). The difference in affinity at the sensor surface and in solution is ascribed to the above mentioned avidity effect due to the formation of GST dimers.[15]

The shift in pK_a value upon binding is linked to the difference in free binding energy (ΔG_b^0) for the -1 and -2 state. At low pH (pH <3) the pY peptide will exclusively bind in the -1 state, in this pH range equation 8 (see Experimental) will transform to

$$K_{obs} = K^{-1} = K^{-2}.10^{pK^b - pK^f} \quad (1)$$

in which K^{-1} is the binding constant for the peptide in the -1 ionisation state. Based on the relation $\Delta G_b^0 = -RT\ln K_a$ a pK_a shift of 1.35 corresponds to a more favourable ΔG_b^0 for the -2 state compared to the -1 state of 1.8 kcal/mol.

Based on the X-ray structure of Lck SH2 in complex with the hmT peptide (PDB entry 1lcj),[16] the pK_a value of the pY peptide bound to the Lck SH2 active site was calculated. Finite difference calculations predict an effective pK_a of 5.3 for the phosphotyrosine residue in the complex, which is in reasonable agreement to the results from the proton linkage analysis of the experimental data.

Discussion

The pK_a for the bound complex (pK^b) for Lck SH2 reported here is significantly larger than the value of 4.0 for pY peptide binding to the phospholipase C-γ_1 C-terminal SH2 domain.[9] This difference may be due to the larger number of basic amino acids in the tyrosine phosphate binding pocket of the phospholipase C-γ_1 C-terminal SH2 domain (four arginines) compared to the Lck SH2 domain (two arginines and one lysine). The pY binding pockets of Lck and Src are rather similar, the value for pK^b determined from SPR is close

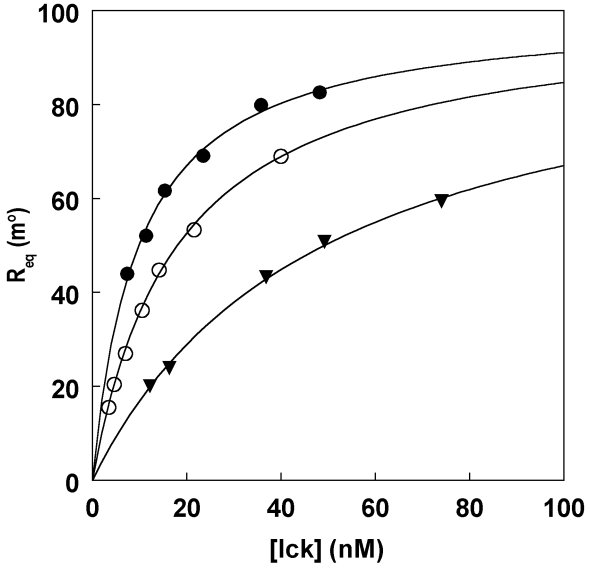


Figure 3. Binding isotherms of the interaction of Lck SH2 with immobilised hmT peptide. R_{eq} for the interaction at the sensor surface is assayed in the presence of various Lck SH2 concentrations. Experiments in triple buffer at 25 °C: pH 9.0 ○; pH 6.8 ●; pH 5.0 ▼. The lines are the fits according to equation 2, from which the affinity at the sensor surface (K_C) is obtained. N.B. For ease of comparison the curves are normalised to a maximum binding signal (R_{max}) of 100 m°.

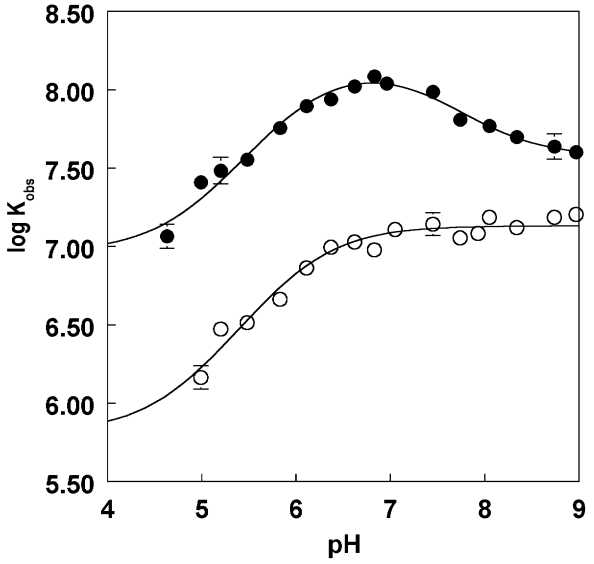


Figure 4. pH dependence of the binding constant (K_{obs}) for the interaction between hmT peptide and Lck-SH2. Experiments in triple buffer at 25 °C. K_{obs} at the sensor surface (K_C, closed symbols) and in solution (K_S, open symbols). The lines are fits according to a two proton-linkage model for K_C (eq 9), and a one-proton linkage model for K_S (eq 8).

Table 1. Protonation and binding parameters derived from proton linkage models

	Interaction at the sensor surface(two-proton linkage model)	Interaction in solution(one-proton linkage model)
$K^{-2}(M^{-1})$	$1.46(\pm 0.09) \times 10^8$	$1.35(\pm 0.06) \times 10^7$
pK^b	4.8 ± 0.1	4.7 ± 0.1
pK_2^f	7.4 ± 0.1	—
pK_2^b	8.0 ± 0.1	—

Analysis of the data in Figure 4. The interaction in solution was analysed with a one-proton linkage model (eq 8), the interaction at the sensor surface with a two-proton linkage model (eq 9). In these models the experimental value of 6.05 for pK^f was applied. Values are presented ± standard errors as indicated by the fit program. Symbols for parameters are as described in Experimental.

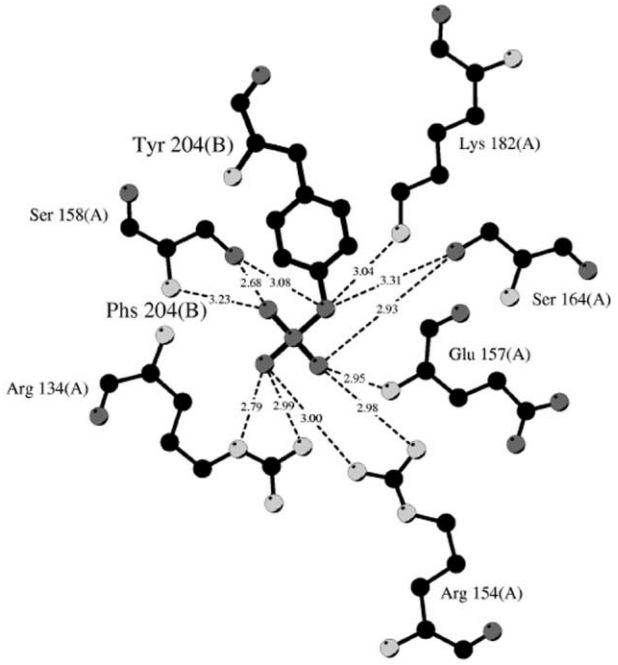


Figure 5. Schematic representation of interactions of the tyrosine phosphate group with residues in the pY binding pocket of Lck SH2. From X-ray structure PDB entry 1LCJ.[15] Distances are indicated between non-hydrogen atoms.

to value of 4.4 found for Src SH2 determined with ITC.[10] In Lck, the threonine in the BC2 position is replaced by a serine, involved in hydrogen bonding to two phosphate oxygens (Ser158, Fig. 5). It is not expected that this will significantly influence the pK_a shift of the pY peptide upon binding. Another difference in the pY binding pocket is that the cysteine in position βC3 in the Src domain is replaced by serine (Ser164) in the Lck domain and other SH2 domains of the Src family. This serine is involved in hydrogen bonding to two of the oxygen atoms of the phosphate group (Fig. 5). The cysteine in the Src SH2 may have a pK_a value around 8 to 9. Bradshaw and Waksman[10] reported a pH effect in the region of 7.5 to 9 for the interaction with the Src SH2 domain, corresponding to a second proton linkage with a pK_a shift from 8.2 for the free to 8.5 for the bound state. They tentatively assigned this pK_a shift to the Cys βC3, which is part of the pY binding pocket. Interestingly, with Lck SH2, which lacks the cysteine residue, we do not observe an effect at pH >7 (Fig. 2) for the interaction in solution, consistent with the assignment by Bradshaw and Waksman.

In view of the generally large inaccuracy of calculated pK_a values, the calculated pK^b value of 5.3 is in reasonable agreement with the experimental value The root-mean-square accuracy of this method is in the order of 0.9 pK_a units, as established in a test with a set of globular proteins.[11] The slight underestimation of the size of the shift is probably a consequence of the high protein dielectric constant which was used in the calculation. A more detailed charge model and a lower protein dielectric constant may give more accurate results if an explicit treatment of tautomerism is included.[17]

Conclusion

This study demonstrates that SPR competition experiments can give reliable results, not influenced by effects at the sensor surface. To the best of our knowledge this is the first report of the use of such SPR competition experiments to assay pH dependence of affinity. This approach is an alternative for ITC assay: With ITC especially high affinity values ($K_D < 10^{-8}$ M) can not be accurately assayed. Furthermore interpretation of the observed enthalpy changes as function of pH is complicated due to different enthalpic effects arising from the exchange of protons with the various buffer components.[10]

Binding of the pY ligand to the Lck SH2 domain shifts the pK_a of the tyrosine phosphate group 1.3 units downwards. This implies that the binding constant of the peptide in the -2 ionisation state of the phosphate group is approximately 20-fold larger than that for the peptide in the -1 state. The highly positively charged binding pocket for the tyrosine phosphate group is responsible for this. When designing mimics for the metabolically labile tyrosine phosphate group one should bear in mind this preferred ionisation state, Mimics with single negative charges will not have optimal affinity. There are several examples of phosphotyrosine replacement by monocharged mimics. For example, Tong et al.[18] reported a 40-fold lower affinity for Lck SH2 upon replacement of pY by carboxymethyl-phenylalanine, although the X-ray structure shows that this mimic maintains similar interactions with the SH2 domain as the pY residue.

Experimental

The Lck SH2 domain was obtained as a GST-fusion protein from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The protein contains the amino acids 120–226 of the Lck kinase of mouse origin. The synthesis of the peptide derived from the hmT antigen with sequence EPQpYEEIPIYL-NH_2, and of a peptide with the same sequence, extended with a N-terminal 6-aminohexanoic acid (Ahx) moiety, to introduce a spatial linker between the SPR sensor matrix and the immobilised pY-ligand, has been described previously.[13]

SPR experiments

SPR experiments were performed with an IBIS II instrument (IBIS technologies, Enschede, The Netherlands) equipped with a CM5 chip (BIAcore). Coupling of the Ahx-hmT peptide to the chip was performed as described previously.[13] The sample cell contained the immobilised Ahx-hmT peptide, and the reference cell was treated identically except that no peptide was added. The net SPR signal is the difference in SPR angle, expressed in m°, between sample and reference cells. SPR experiments at 25 °C in a pH range of 4.5–9 were performed in triple buffer containing 10 mM sodium acetate, 10 mM 4-morpholineethanesulfonic acid (MES), 20 mM tris(hydroxymethyl)aminomethane

(TRIS), 150 mM sodium chloride, 3.4 mM EDTA and 0.0005% tween-20. Immediately before use 4 mM dithiothreitol was added. Buffers were adjusted to proper pH with 6 N hydrochloric acid. To avoid contamination of the interaction buffer with running buffer and pH changes of the sample, immediately before each experiment the cells were washed with the appropriate interaction buffer, using automatic procedures provided by the IBIS autosampler

Analysis of SPR data

The binding constant for the interaction at the sensor surface (K_C) was determined as previously described,[13] by fitting of the net SPR signal at equilibrium (R_{eq}) as function of [SH2] according to a binding isotherm:

$$R_{eq} = \left(\frac{[SH2]}{[SH2] + K_C}\right) \times R_{max} \quad (2)$$

in which R_{max} is the net SPR signal at maximum binding capacity of the sensor chip.

The binding constant for the interaction in solution (K_S) was determined from experiments with a fixed [SH2] in the presence of various concentrations of competing pY peptide, based on the approach described by Morelock et al.[15] K_S is defined as a binding constant, which in principle is independent from effects at the sensor surface:

$$K_S = \frac{[pY].[SH2]}{[pY - SH2]} \quad (3)$$

in which [pY-SH2] is the concentration of the complex SH2 domain with pY peptide. In the presence of competing peptide the free [SH2] will decrease, and eq 2 will change to

$$R_{eq} = \left(\frac{[SH2]_{tot} - [pY - SH2]}{[SH2]_{tot} - [pY - SH2] + K_C}\right) \times R_{max} \quad (4)$$

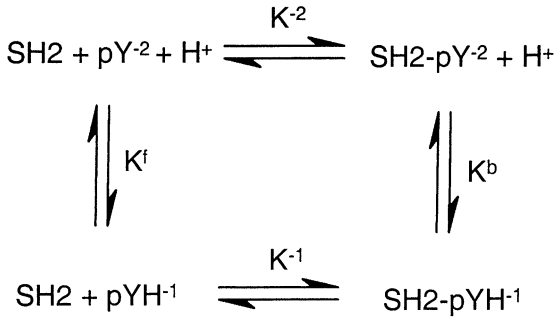
[pY-SH2] is a quadratic function of K_S; it can be derived that

$$[pY - SH2] = \frac{\left(K_S + [SH2]_{tot} + [pY]_{tot}\right) - \sqrt{\left(K_S + [SH2]_{tot} + [pY]_{tot}\right)^2 - 4 \times [SH2]_{tot} \times [pY]_{tot}}}{2} \quad (5)$$

in which $[pY]_{tot}$ and $[SH2]_{tot}$ are the total concentration of pY peptide and SH2 domain in the experiment. Substitution of eq 5 into eq 4 yields an expression describing the inhibition curves (R_{eq} vs [pY]), from which K_S and R_{max} can be obtained by fitting the curve.

Proton linkage models

The pH-dependence of K_S and K_C was analysed with proton-linkage models for the binding. In the scheme the steps are shown for binding of pY peptide to the SH2 domain, involving protonation of the tyrosine phosphate group.



K^{-1} and K^{-2} are the binding constants for binding to the SH2 domain of the pY peptide in the -1 and -2 ionisation state, respectively. K^f and K^b are the proton binding constants for the free and bound pY peptide, respectively. The reactions in the scheme form a thermodynamic cycle, from which it follows that

$$K^{-2} \times K^b = K^{-1} \times K^f \quad (6)$$

Equation 6 links the proton binding constants to the ligand binding constants. Depending on pH, the peptide binds in the -2 as well as -1 state, resulting in complex SH2-pY^{-2} and SH2–pYH^{-1}, respectively. The binding constant at a given pH (K_{obs}) to which both species contribute can be defined as

$$K_{obs} = \frac{\left[SH2 - pY^{-2}\right] + \left[SH2 - pYH^{-1}\right]}{[SH2] \times \left(\left[pY^{-2}\right] + \left[pYH^{-1}\right]\right)} \quad (7)$$

Combining eqs 6 and 7 yields an expression for K_{obs}:

$$K_{obs} = K^{-2}.\frac{1 + 10^{pK^b - pH}}{1 + 10^{pK^f - pH}} \quad (8)$$

Fits of K_{obs} versus pH using eq 8 give values for K^{-2}, pK^b and pK^f. The scheme above can be extended to two protonation events linked to binding. For example if protonation of the protein and protonation of the ligand is linked to binding, eq 9 can be derived[10]

$$K_{obs} = K^{-2}.\frac{1 + 10^{pK^b - pH} + 10^{pH - pK_2^b}}{1 + 10^{pK^f - pH} + 10^{pH - pK_2^f}} \quad (9)$$

in which pK_2^b and pK_2^f are the pK_a values for protonation of the protein in bound and free state, respectively.

Assay of pK_a of the free phosphotyrosine peptide

A solution of approximately 0.5 mM of the hmT peptide in triple buffer was made and titrated with steps of 2 μL 3N HCl. After each addition the pH and the absorbance at 268 nm were recorded. The absorption was corrected for the small degree of dilution during titration. The pK_a value of the free pY peptide (pK^f) was obtained from a fit of the absorbance versus pH data to eq 10.

$$\mathrm{Abs} = \mathrm{Abs1} + \frac{\mathrm{Abs2} - \mathrm{Abs1}}{1 + 10^{\mathrm{p}K^f - \mathrm{pH}}} \quad (10)$$

with Abs1 and Abs2 the absorbance at pH $<<$ pK_a and pH $>>$ pK_a, respectively.

Curve fitting

Nonlinear curve fitting according to specified models was performed with SlideWrite Plus for Windows, version 4, from Advanced Graphics Software Inc. (Carlsbad, CA, USA).

pK_a calculations

The crystal structure of Lck SH2 domain with bound 11-mer hmT peptide (PDB entry 1lcj; resolution 1.8 Å)[16] was used as input to the single titration site pK_a calculation[11,12] procedure of the UHBD package (version 5.1).[19] All bound water molecules were removed from the structure. The phosphotyrosine residue was included in the calculation with possible ionisation states of −2 and −1 and a model compound pK_a of 5.8. Atomic charges were taken from the CHARMM22 parameter set and atomic radii from the OPLS parameter set.[20,21] Hydrogen atoms were added to the model using the HBUILD procedure of CHARMM (version 27b3). A dielectric constant of 80 was used for bulk water, and 20 for the protein interior. All titrating groups of the protein and peptide were included in the calculation.

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