pH Titration studies of an SH2 domain–phosphopeptide complex: Unusual histidine and phosphate pK_a values

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Abstract

Electrostatic interactions in a complex of the phospholipase C- γ C-terminal SH2 domain with a high-affinity binding phosphopeptide representing the sequence around Tyr 1021 of the β platelet-derived growth factor receptor were studied by pK_a determination of various titratable groups over the pH range of 5 to 8. A histidine residue that is highly conserved among SH2 domains (His β D4) and the phosphotyrosine (pTyr) phosphate group show pK_a values significantly lower than average for these residue types in proteins. The reduced pK_a of these two groups is due to the proximity of the highly positively charged pTyr binding pocket. The unusual pK_a of His β D4 is also due to burial from solvent in a hydrogen-bonding network that appears necessary for the positioning of arginine residues involved in pTyr binding. Mutation of the analogous histidine in other SH2 domains has been shown to abrogate pTyr binding. In addition to these large shifts in pK_a values, smaller effects were observed for the titratable groups of a glutamic acid and histidine near the C-terminus of the second helix due to its helical dipole. Finally, exchange behavior of arginine guanidinium protons with solvent as a function of pH in this SH2 domain–phosphopeptide complex confirms previous descriptions of the roles of different arginines in the structure and function of this protein.

Keywords: histidine; NMR; phospholipase C- γ ; phosphotyrosine; pK_a determination; SH2 domain

Electrostatic interactions in proteins are very important in catalysis, ligand recognition processes, and protein stability (Gilson, 1995). We are interested in the electrostatic interactions of Src homology 2 (SH2) domains with their phosphotyrosine-containing targets. SH2 domains are small (~ 100 amino acid) domains that play an important role in signal transduction by binding to specific sequences on growth factor receptors or other molecules containing phosphotyrosine (for a review, see Schaffhausen, 1995). Studies of the binding of SH2 domains to their target proteins have been facilitated by modeling of the interacting region of the target protein with small (~ 20 amino acids or less) phosphopeptides containing sequences around the site of Tyr phosphorylation. These peptides can significantly inhibit the biological interaction (Yonezawa et al., 1992; Larose et al., 1993; Nishimura et al., 1993). SH2 domain binding to phosphopeptides is characterized by a very rapid (diffusion-limited or faster) on-rate (Panayotou et al., 1993), most likely due to electrostatic attraction of the phosphotyrosine (pTyr) phosphate groups with the highly positively charged binding pockets observed in the structures of SH2 domains and SH2 domain–phosphopeptide complexes solved to date (Eck et al., 1993; Waksman et al., 1993; Gilmer

et al., 1994; Lee et al., 1994; Pascal et al., 1994, 1997; Narula et al., 1995; Xu et al., 1995; Nolte et al., 1996). This basic pocket is particularly evident in the phospholipase C- γ C-terminal SH2 (PLCC SH2) domain, which contains four arginine residues in the pTyr binding region. We have previously determined the solution structure (shown schematically in Fig. 1) of this domain in complex with a high-affinity binding phosphopeptide (pY1021) representing sequences around Tyr 1021 of one of its biological targets, the β platelet-derived growth factor receptor, and have investigated the dynamic properties of the complex (Farrow et al., 1994; Pascal et al., 1994, 1995; Kay et al., 1996). Our studies and other structures of SH2 domain–peptide complexes have highlighted the significance of the electrostatic contribution to peptide binding via extensive charge–charge and hydrogen-binding interactions to the phosphate of the pTyr. To better understand the role of electrostatic interactions in SH2 domain-mediated recognition, we have performed experimental pK_a determinations of ionizable groups in the PLCC SH2 domain–pY1021 complex over the pH range of 5 to 8.

NMR has been shown to be very useful for studying electrostatic interactions because pK_a values of ionizable groups in proteins can be determined by following chemical shifts as a function of pH. The chemical shift is dependent on through-space effects of titratable groups, which have a $1/r^2$ dependence (Asakura et al., 1992). Thus, the effect of ionizable groups on chemical shift can be

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Fig. 1. Ribbon diagram of the PLCC SH2 domain-pY1021 complex highlighting particular residues described in this study with the secondary structural elements labeled using the nomenclature described in Eck et al. (1993). This notation gives the secondary structural element with the residue numbered from the beginning of that element (i.e., $\alpha A2$ or $\beta D6$). Loops are named by the two secondary structural elements between which they occur (i.e., CD loop between the βC and βD strands). Figure drawn using the program MOLSCRIPT (Kraulis, 1993).

quite long range, so that a particular chemical shift change may be due to contributions from multiple titratable groups. For this reason, it is preferable to probe chemical shift changes of the nucleus that is directly involved in the titration or of a nucleus that is only one-bond separated from the titratable group. Examples include changes in ^{31}P chemical shifts to study phosphate pK_a s (Vogel, 1989), $^{15}\text{N}_{\delta 1}$, and $^{15}\text{N}_{\epsilon 2}$ chemical shifts of histidine imidazole groups (Bachovchin, 1986; van Dijk et al., 1990, 1992; Lodi & Knowles, 1991; Annand et al., 1993; Pelton et al., 1993; Tsilikonas et al., 1996) and ^{13}C side chain carboxyl chemical shifts for aspartic and glutamic acid residues (Jeng & Dyson, 1996; Qin et al., 1996).

In this study, we have measured ^{31}P chemical shifts to investigate the effect of the electrostatic contribution to pTyr binding on its phosphate pK_a . Histidine imidazole pK_a values have also been determined over this pH range using imidazole $^{15}\text{N}_{\delta 1}$ chemical shift changes. Within the PLCC SH2 domain construct are five histidine residues, most of which are poorly conserved among SH2 domains. One highly conserved histidine in the PLCC SH2 domain is His 57 or His $\beta D4$ in the nomenclature described in Eck et al. (1993). This notation, common in the description of SH2 domain structures, will be used for conserved residues among SH2 domains and is explained briefly in the legend to Figure 1. Mutation of the conserved His $\beta D4$ in the GAP N-terminal SH2 domain to a glutamic acid or lysine destroys *in vitro* binding to tyrosine-phosphorylated proteins (Marangere & Pawson, 1992), and mutation of the homologous residue in the adapter protein drk to an isoleucine disrupts its *in vivo* role in *Drosophila* eye development (Olivier et al., 1993). However, in the solution structure of the PLCC SH2 domain-pY1021 complex, NOEs were

observed between the pTyr aromatic protons and the His $\beta D4$ α and β protons but not the imidazole ring (Pascal et al., 1994). This lack of contact between the His $\beta D4$ ring and the pTyr has also been observed in other SH2 domain-phosphopeptide complexes, solved both by heteronuclear NMR techniques and X-ray crystallography (Eck et al., 1993; Waksman et al., 1993; Gilmer et al., 1994; Lee et al., 1994; Pascal et al., 1994; Xu et al., 1995). Thus, His $\beta D4$ is both highly conserved among SH2 domains and necessary for binding function; however, no direct interaction with the pTyr or other ligand residues is present. Residues without direct contact to a ligand can modify binding energy via electrostatic effects, as is well understood. Nonetheless, it is of interest to define the specific interactions that mediate these effects. In this study, the observation of an unusual pK_a for this residue is presented as evidence that His $\beta D4$ forms hydrogen bonds with the arginine of the highly conserved FLVR sequence, Arg 37 or Arg $\beta B5$, and positions this residue for pTyr recognition.

Results and discussion

Histidine pK_a values in the SH2 domain-peptide complex

Although ^1H - ^{15}N HMBC experiments and their use for histidine pK_a determinations have been described previously (Pelton et al., 1993), we provide a brief summary of the features of these spectra due to the importance of the data for our results. The HMBC experiments used in this study differ significantly from one-bond ^1H - ^{15}N HSQC experiments. Observation of the protons directly attached to ^{15}N atoms on the imidazole ring is very difficult because they may be absent (for one of the two nitrogens in the neutral state) or in very rapid exchange with solvent. (See the lower left corner of Fig. 2 for a diagram of the histidine imidazole ring.) Thus, carbon-bound protons that are two- or three-bond correlated with the ^{15}N atoms are observed. The chemical shift of the ^{15}N resonance is strongly correlated to the protonation state of the imidazole nitrogen atom with protonated nitrogens resonating at ~ 170 ppm and deprotonated nitrogens at ~ 250 ppm. One can also differentiate between the two neutral tautomers using these experiments because coupling constants differ, depending on the influence of the lone pair of electrons on the unprotonated ^{15}N atom (Blomberg et al., 1977).

The complete assignment of the histidine imidazole side-chain resonances has been performed previously (Yamazaki et al., 1993). Regions of ^1H - ^{15}N HMBC spectra showing the two- and three-bond ^1H , ^{15}N correlations for histidine residues at pH 5.4, 6.6, and 7.8 are shown in Figure 2, with a diagram of the histidine imidazole ring shown in the lower left corner. The five histidine residues of the SH2 domain have well-dispersed resonances. At pH 7.3, the resonances from His 13 are broadened beyond detection, and at pH 8.0, resonances of His 23 and His 87 are significantly broadened, probably due to exchange with solvent. The $^{15}\text{N}_{\delta 1}$ and $^{15}\text{N}_{\epsilon 2}$ resonances are clearly resolved with distinct chemical shifts, particularly at higher pH values where the imidazole ring approaches the neutral state. One exception to this is His 87, for which the two imidazole ^{15}N chemical shifts are very similar throughout the titration, and identical between pH 6.6 to 7.0. Because the original assignment of the histidine residues was performed at pH 5.7, the identities of the $^{15}\text{N}_{\delta 1}$ and $^{15}\text{N}_{\epsilon 2}$ resonances of His 87 were ambiguous above pH 7.0. This ambiguity can be resolved by requiring that the titration curves for both the $^{15}\text{N}_{\delta 1}$

Fig. 2. The histidine $H_{\delta 2}/H_{\epsilon 1}-N_{\delta 1}/N_{\epsilon 2}$ region of 1H - ^{15}N HMBC spectra of the PLCC SH2 domain-pY1021 complex at pH 5.4 (A), 6.6 (B), and 7.8 (C). Correlations for all five histidines are observed at lower pH. At higher pH, resonances of His 13 are broadened beyond detection due to exchange. A diagram of the histidine imidazole ring is shown in the lower left corner. Note that neither of the two labile protons that may be present ($H_{\delta 1}$ or $H_{\epsilon 2}$) are included.

and $^{15}N_{\epsilon 2}$ resonances be sigmoidal and that both give identical pK_a values (data not shown). As described above, imidazole ^{15}N resonances cluster about 170 ppm or 250 ppm in the protonated or unprotonated states, respectively (Pelton et al., 1993). Intermediate values of these ^{15}N resonances at higher pH are indicative of fast exchange between the two neutral tautomers (Van Dijk et al., 1992). Using a value of 250 ppm for the chemical shift of the non-protonated resonance for either of the two neutral tautomers, and 170 ppm as a value for the chemical shift of the protonated ^{15}N resonance (Pelton et al., 1993), it would be expected that an imidazole ring with an equal population of the $^{15}N_{\epsilon}$ - and $^{15}N_{\delta}$ -protonated neutral tautomers would yield an average chemical shift of 210 ppm. Based on the fit of data for the chemical shift of the His 87 $N_{\delta 1}$ resonance, the chemical shift in the neutral state is 207.6 ± 0.3 . Thus, His 87 appears to be approximately 50% populated by each tautomer under high pH conditions that stabilize the neutral state. By extension, at lower pH there could be an equilibrium between the protonated and both neutral tautomeric states. The $^{15}N_{\delta 1}$ resonance of His 23 was also upfield of 250 ppm at the end of the titration, and by the same procedure it was estimated to have approximately 75% of the N_{ϵ} -protonated tautomer (see Fig. 3) at high pH. However, these estimates of the percentage of the two tautomers are subject to error caused by secondary effects on the histidine $^{15}N_{\delta 1}$ chemical shift due to ring current effects and hydrogen bonds, particularly because His 87 contacts aromatic rings in the βF strand (Phe 74) and αB helix (Tyr 83 and Tyr 84) (Pascal et al., 1997). Nonetheless, the presence of conformational exchange between the two tautomeric states is clear from the general trends of the chemical shift values.

Figure 3 shows the chemical shift changes for the $^{15}N_{\delta 1}$ resonances of histidines as a function of pH with the pK_a values calculated as described below (Materials and methods). Free histidine

has a pK_a of 6.0 (Tanokura, 1983), but modification of the histidine zwitterion by N-acetylation and carboxymethylation (Tanokura, 1983) or formation of peptide bonds results in the imidazole ring having a pK_a value of 6.3–6.4 (Nozaki & Tanford, 1967). The pK_a of His 23 is identical to this value, not surprising since this residue is on the solvent-exposed face of helix αA in calculated structures. The pK_a of His 13, 5.9, is shifted slightly lower than this value, which may be due to its proximity to Arg 37 and Arg 39 (Arg $\beta B5$

Fig. 3. Histidine $^{15}N_{\delta 1}$ chemical shifts for the PLCC SH2 domain-pY1021 complex as a function of pH with calculated pK_a values. Solid lines represent the best fit to the data. Given the chemical shifts of the $N_{\delta 1}$ and $N_{\epsilon 2}$ resonances of His 57 and the fact that they are unchanged over the pH range studied, the His 57 pK_a value must be less than 4.0.

and $\beta B7$) in the highly positively charged pTyr-binding site. The pK_a of His 6 at 5.6 is shifted even lower. This residue is five residues N-terminal to Trp 11, the first residue in strand βA often thought to mark the beginning of the folded domain, but has been shown in the structure of this SH2-phosphopeptide complex (Pascal et al., 1997) and at least one other SH2-phosphopeptide complex (Nolte et al., 1996) to pack against hydrophobic residues in the N-terminus of helix αB . Thus, the electrostatic field due to helix αB may contribute to the lower pK_a of His 6. In contrast, the pK_a of His 87 at 7.0 is significantly higher than the value for histidine in random coil peptides and proteins. His 87 is at the C-terminus of the αB helix, and the electrostatic field due to the helix dipole could contribute to this higher pK_a value (Lodi & Knowles, 1993).

The most dramatic perturbation from the pK_a of free histidine was exhibited for His 57 or His $\beta D4$, whose $^{15}N_{\delta 1}$ and $^{15}N_{\epsilon 2}$ chemical shifts remain unchanged over the course of the titration at values characteristic of the neutral N_{ϵ} -protonated tautomer. Thus, the pK_a of this residue must be less than 4.0. There are three explanations for this anomalous behavior. The presence of the strong positive electrostatic potential in the pTyr-binding site could lower the pK_a of His $\beta D4$. Partial or complete burial of the side chain in a hydrophobic environment could also lower the pK_a (Del Buono et al., 1994). Finally, specific hydrogen-bonding interactions could stabilize the neutral N_{ϵ} -protonated tautomer. His $\beta D4$ is a very highly conserved residue among SH2 domains, and mutation of this residue has been shown to affect SH2 domain function in vitro (Marangere & Pawson, 1992) and in vivo (Olivier et al., 1993). In the crystal structures of phosphopeptide complexes of the Src (Waksman et al., 1993; Gilmer et al., 1994), Lck (Eck et al., 1993), and N-terminal Syt (Lee et al., 1994) SH2 domains, the $N_{\epsilon 2}$ atom is completely buried, and this has also been observed in refined structures of the PLCC SH2 domain-pY1021 complex (Pascal et al., 1997). In these crystal structures, the His $\beta D4$ imidazole ring is involved in a network of hydrogen-bonding interactions involving the $H_{\epsilon 2}$ atom with the Glu $\alpha A6$ carboxylate group, and the $N_{\delta 1}$ atom with the η protons of Arg $\beta B5$ (see Fig. 4). The O_{γ} of Ser $\beta C5$ also participates in this network, accepting a hydrogen bond from the H_{ϵ} proton of Arg $\beta B5$. These four residues are all highly conserved among SH2 domains (Marangere & Pawson, 1992). The His $\beta D4$ residue may thus play a critical role in positioning the Arg $\beta B5$ residue for optimal binding interactions to pTyr through this hydrogen-bonding network, which requires the $N_{\delta 1}$ atom to be deprotonated and the $N_{\epsilon 2}$ atom to be protonated. NOEs from the $H_{\epsilon 1}$ proton to protons of Ser $\beta C5$, Arg $\beta B5$, and Glu $\alpha A6$ have been observed in the spectra of the PLCC SH2 domain-pY1021 complex (Pascal et al., 1997), consistent with preservation of this hydrogen-bonding network. Of note is that the pH dependence of the binding of phosphopeptides to the Lck SH2 domain has also been interpreted in terms of compromising this hydrogen-bonding network, which optimizes binding site geometry (Lemmon & Ladbury, 1994).

Histidine pK_a values for the free PLCC SH2 domain

If the interactions involving His $\beta D4$ play a role in orienting Arg $\beta B5$ to facilitate pTyr binding, then these hydrogen bonds might also be expected in the free PLCC SH2 domain to facilitate the rapid binding of ligand. In this case, a lowered pK_a for His $\beta D4$ in the free protein should also be observed. A 0.2 mM sample of the PLCC SH2 domain in 100 mM sodium acetate (phosphate-free) was used to study the pK_a of this histidine in the free PLCC SH2

Fig. 4. The hydrogen-bonding network involving His $\beta D4$ and pTyr, showing the positions of the side chains of Glu $\alpha A6$, Arg $\beta B5$, Ser $\beta C5$, and His $\beta D4$ of the Syt N-terminal SH2 domain, as well as hydrogen bonds calculated for this structure (from the phosphopeptide complex structure with PDB filecode 1AYA; Lee et al., 1994). The hydrogen bonds stabilize the N_{ϵ} -protonated neutral tautomer for the His $\beta D4$ imidazole ring and position the Arg $\beta B5$ guanidinium group for pTyr binding. Structures were visualized and hydrogen bonds were calculated using Quanta v4.0 (Molecular Simulations Inc., 1993).

domain to remove any possible effects of phosphate complexation of the SH2 domain to the His $\beta D4$ pK_a . Previous studies (Waksman et al., 1993; Pascal et al., 1995) have shown that free phosphate ions bind to SH2 domains. The phosphate binding site is very close to the His $\beta D4$ imidazole ring.

Regions of 1H - ^{15}N HMBC spectra for the free PLCC SH2 domain in 100 mM sodium acetate, pH 5.0, and the ^{15}N PLCC SH2 domain-pY1021 complex in 50 mM sodium acetate, 50 mM sodium arsenate, pH 5.0, are shown in Figure 5. These two spectra are very similar, indicating similarity in the electrostatic behavior of the two samples and likely similar pK_a values. In particular, spectra show that His $\beta D4$ is present in the N_{ϵ} -protonated tautomer in both the free and peptide-complexed states. Note that assignment of this residue in the free SH2 domain was made by inspection based on the similarity between the spectra of free and complexed SH2 domain.

The triad of hydrogen bonds involving Glu $\alpha A6$, Ser $\beta C5$, and His $\beta D4$ has been observed in the crystal structures of SH2 domain-phosphopeptide structures that we have analyzed, including the N-terminal SH2 domain of the Syt phosphatase (Lee et al., 1994) and SH2 domains of Lck (Eck et al., 1993) and Src (Waksman et al., 1993). With respect to crystal structures of uncomplexed SH2 domains, the situation appears more variable. In the crystal structure of the N-terminal SH2 domain of Syt (Lee et al., 1994), all three hydrogen bonds are observed. However, in the SH2 domain of Src (Waksman et al., 1993), the presence of these hydrogen bonds differs among the four molecules that make up the asymmetric unit. In two of these molecules, all three hydrogen bonds are observed. In one of the others, the His $\beta D4$ $H_{\epsilon 2}$ /Glu $\alpha A6$ O_{δ}

Fig. 5. The histidine $H_{\delta 2}/H_{\epsilon 1}-N_{\delta 1}/N_{\epsilon 2}$ region of 1H - ^{15}N HMBC spectra of the (A) free PLCC SH2 domain in 100 mM sodium acetate, pH 5.0 and (B) the PLCC SH2 domain-pY1021 complex at pH 5.0 in buffer containing 50 mM sodium acetate and 50 mM sodium arsenate.

distance is 2.9 Å, a distance that would permit only a weak hydrogen bond. In the other, the conformation of Arg $\beta B5$ is altered such that different Arg $\beta B5$ H_{γ} protons make contact with the Ser $\beta C5$ O_{γ} and His $\beta D4$ $N_{\delta 1}$ than shown in Figure 4. Finally, these hydrogen bonds are poorly conserved in the two SH2 domains in the crystallographic dimer observed in the structure of Grb2 (Maignan et al., 1995). In one, the distance between His $\beta D4$ $H_{\epsilon 2}$ and Glu $\alpha A6$ O_{δ} atoms is 3.4 Å, too far for hydrogen bonding, but the other two hydrogen bonds are present. In the other, there is a dramatic change from the hydrogen-bonding geometry shown in Figure 4, with Arg $\alpha A2$ making a salt bridge to the Glu $\alpha A6$ carboxyl group and His $\beta D4$ no longer in contact with either Arg $\alpha A2$ or Glu $\alpha A6$. The reason for the altered conformation in this SH2 domain of Grb2 is unclear, but interactions due to packing of the asymmetric units in the crystal bring a pair of basic residues from the N-terminal SH3 domain in one molecule to within 5 Å of the phosphate-binding pocket in the other.

One possible explanation for the variability of these hydrogen bonds in free SH2 domains is the solvent accessibility of the Glu $\alpha A6$ O_{δ} and His $\beta D4$ $N_{\epsilon 2}$. Peptide binding partially buries the Glu $\alpha A6$ O_{δ} and almost completely buries the His $\beta D4$ $N_{\epsilon 2}$. Thus, it is not surprising that the His $\beta D4$ $H_{\epsilon 2}$ to Glu $\alpha A6$ O_{δ} hydrogen bond is the least conserved in free SH2 domains. Since the $N_{\delta 1}$ of the His $\beta D4$ imidazole ring is always buried in the protein interior, the hydrogen bond to Arg $\beta B5$ is much less variable. The hydrogen bonds involving the His $\beta D4$ $N_{\delta 1}$, Ser $\beta C5$ hydroxyl and Arg $\beta B5$ guanidinium appear to be more important than the His $\beta D4$ $H_{\epsilon 2}$ and Glu $\alpha A6$ O_{δ} hydrogen bond in stabilizing the position of the Arg $\beta B5$ guanidinium group and maintaining the N_{ϵ} -protonated tautomer of His $\beta D4$. The fact that in the free PLCC SH2 domain the His $\beta D4$ exists in the neutral N_{ϵ} -protonated tautomer at physiologic pH values with a low pK_a value suggests that interactions

stabilizing this state are maintained in the absence of phosphotyrosine ligands, most likely including the His $\beta D4$ $N_{\delta 1}$ to Arg $\beta B5$ H_{γ} hydrogen bond.

Phosphotyrosine phosphate pK_a

Free phosphotyrosine has a pK_a of 5.8 for the transition from the -2 to -1 state (Vogel, 1989). In Figure 6, the ^{31}P chemical shifts of the pTyr phosphate groups in the free pY1021 phosphopeptide and the PLCC SH2 domain-pY1021 complex as a function of pH with the calculated pK_a values are shown. A pK_a value of 6.1 is observed for the free pY1021 phosphate group. Because the chemical shift of the ^{31}P signal of the pTyr in the PLCC SH2 domain-pY1021 complex is unchanged between pH 5.3 and 7.5, the pK_a must be shifted by more than one pH unit upon binding to the PLCC SH2 domain. Using the ^{31}P chemical shift of the -1 state for the free peptide to estimate the chemical shift of the -1 state for the pTyr in the PLCC SH2 domain complex, we obtain a pK_a value of 4.0.

This shift in pK_a can be explained by the stabilization of the -2 state in the highly basic pTyr binding pocket of the SH2 domain. However, a similar change of pK_a has not been observed for the Src SH2 domain-pYEEI complex, in which a pK_a of 5.5 was measured, compared to a value of 5.9 for the free peptide (Xu et al., 1995). This difference may be due to the greater number of basic amino acids in the pTyr binding pocket of the PLCC SH2 domain (four arginines) than in the pTyr-binding region of the Src SH2 domain (two arginines and one lysine). This result is significant, because the different electrostatic potentials in the pTyr binding sites of different SH2 domains may contribute to their specificities indirectly, by a partitioning of the free energy of interaction with different amounts of the binding energy from pTyr contacts and the balance from other interactions. The differential dependence on non-pTyr residues could possibly be exploited to disrupt particular classes of SH2 domain interaction and not others. Aid in the interpretation of this low pK_a value based on computer simulations has been hindered by the difficulty in parameterization of the electrostatic portions of the force fields for pTyr in these protein complexes (Philippopoulos & Lim, 1995).

Fig. 6. Phosphotyrosine ^{31}P chemical shifts for the PLCC SH2 domain-pY1021 complex and the free pY1021 phosphopeptide as a function of pH with calculated pK_a values. Solid lines represent the best fit to the data.

One-bond ^1H - ^{15}N HSQC spectra

Backbone amide ^1H - ^{15}N correlations were used to confirm the histidine $\text{p}K_a$ values determined by the HMBC spectra. It is important to note that the chemical shift changes are much smaller in these HSQC spectra than in the HMBC spectra, with differences in ^{15}N chemical shifts often less than 2 ppm in HSQC spectra compared to 30–70 ppm in the HMBC spectra. This is due to the direct effect of the protonation state on the imidazole ring nitrogen as opposed to the through-space effect of the electrostatic field of the ionizable group on backbone amides. Therefore, the accuracy of the determination of $\text{p}K_a$ values from the HMBC spectra is much greater. However, these spectra can also be used to derive information about cysteine sulfhydryl groups that are ionizable in the pH range of 5 to 8, as well as aspartic or glutamic acid carboxyl groups, which may be protonated at the low end of this pH range. Although sulfhydryl and carboxyl group ionizations would have the greatest effects on the directly bonded Cys $^{13}\text{C}\beta$ or ^{13}C -carboxyl resonances, respectively, chemical shift changes can also be observed for nearby backbone amide ^1H and ^{15}N resonances that are easily detected in ^1H - ^{15}N HSQC spectra of ^{15}N -labeled samples.

Amide ^1H resonances that change by greater than 0.15 ppm and ^{15}N resonances that change by greater than 0.6 ppm in the HSQC spectra recorded over the pH range of 5.0 to 8.0 and that fit a simple sigmoidal curve are provided as supplementary material with calculated $\text{p}K_a$ values. Many of the backbone amide ^{15}N and ^1H resonances from residues in the N- and C-termini undergo significant chemical shift changes as a function of pH, likely due to effects of nearby titrating histidines. Amide resonances of Ile 5 and Lys 9 are strongly affected by titration of the His 6 imidazole group ($\text{p}K_a = 5.6$), resonances of Tyr 12, His 13, and Ala 14 are affected by the His 13 imidazole group ($\text{p}K_a = 5.9$) and resonances of His 23 and Met 24 are affected by the His 23 imidazole group ($\text{p}K_a = 6.3$).

In addition, the effect of pH on resonances of His 23, Met 24, and Leu 25 were useful in assignment. Original assignment of these residues was made at pH 5.8, but these resonances coalesce and then move to new chemical shifts during the course of the titration. Their identities could be established by assuming that the changes in chemical shift fit sigmoidal curves with a $\text{p}K_a$ value close to that previously determined for the imidazole group of His 23.

There is only one cysteine residue in the PLCC SH2 domain (Cys 58 or Cys $\beta\text{D}5$) and none in the sequence of the pY1021 PDGFR phosphopeptide. No significant change in the amide ^1H or ^{15}N chemical shifts of Cys $\beta\text{D}5$ or nearby residues (His $\beta\text{D}4$ and Arg $\beta\text{D}6$) were observed as a function of pH in these HSQC experiments. Resonances of Ala 46, Ile 47, and Ser 48, close in space to the cysteine, also were not perturbed. Thus, the $\text{p}K_a$ of Cys $\beta\text{D}5$ must be 9 or greater, higher than the $\text{p}K_a$ of free cysteine ($\text{p}K_a = 8.3$). This increased $\text{p}K_a$ value is likely due to burial of the sulfhydryl group in the hydrophobic binding interface of the peptide complex. Cys $\beta\text{D}5$ has been shown by structural studies (Pascal et al., 1994) to be involved in phosphopeptide recognition at the +1 position relative to the pTyr leading to burial of the sulfhydryl group. Cys $\beta\text{D}5$ is conserved between the N- and C-terminal SH2 domains of PLC γ , both of which have a strong preference for hydrophobic amino acids at the +1 position (Songyang et al., 1993). In general, the identity of the residue at the $\beta\text{D}5$ position is very important in SH2 domain recognition of phosphopeptides at the +1 position (Songyang et al., 1994).

The carboxyl groups of aspartic and glutamic acid residues have intrinsic $\text{p}K_a$ values of 4.0 and 4.4, respectively (Nozaki & Tanford, 1967). In the PLCC SH2 domain–pY1021 complex, significant changes in ^1H or ^{15}N chemical shifts over the pH range of 5 to 8 were observed for backbone amides of Glu 10, Glu 63, Glu 85, and Glu 101. Changes in chemical shifts of Glu 10 and 101 may be due to titration of His 6 and/or His 13 as titration curves for these residues yield $\text{p}K_a$ values close to 6. However, chemical shift changes of backbone ^1H or ^{15}N resonances of a number of residues in the small β -sheet (strands $\beta\text{D}'$ to βF) and the αB helix could be fit with $\text{p}K_a$ values significantly lower than the $\text{p}K_a$ values of His 6 and His 13. The Glu 85 ^{15}N resonance is affected by an ionizable group that was fit to a $\text{p}K_a$ of 5.3, most likely the Glu 85 carboxyl group itself with a $\text{p}K_a$ value approximately 1 pH unit higher than expected for glutamic acid. Glu 85 lies near the C-terminus of the αB helix and the negative potential due to the helical dipole may raise the $\text{p}K_a$ of the carboxyl group of Glu 85, as suggested previously for the imidazole group of His 87.

Large chemical shift changes for the backbone ^{15}N resonances of Glu 63, Ser 76, and Val 78 are observed over the pH range of 5 to 6. Based on the structure of the PLCC SH2 domain–pY1021 complex, these residues are most likely responding to the sum of the effects of ionization of the carboxyl groups of Glu 63, Glu 73, Glu 75, and Asp 79 and also to the nearby His 6 imidazole group. This region of the PLCC SH2 domain contains a large concentration of negative charge and has been speculated to be a Ca^{+2} binding site (Mahadevan et al., 1994).

Amide exchange with solvent

The rate of amide exchange with water increases as a function of pH from a minimum around pH 3 (Wüthrich, 1986). The lowest rates of exchange are for protons buried in the protein interior or protected by stable hydrogen bonds. The resonances broaden as the rate increases from the slow exchange limit at which sharp peaks can be observed in ^1H - ^{15}N HSQC spectra, to the intermediate exchange limit. Finally, the resonances merge with the water peak in the fast exchange limit. The effect of slow or intermediate exchange with water on the intensity of HSQC peaks can be minimized by using experiments which do not perturb the water resonance (Grzesiek & Bax, 1993; Stonehouse et al., 1994; Zhang et al., 1994) even at pH values slightly above neutral. The arginine side-chain guanidinium resonances were examined as a function of pH to probe the extent to which the different guanidinium side chains are protected from exchange by hydrogen bonding or burial in the PLCC SH2 domain–pY1021 complex. No change in chemical shifts for Arg 18, 37, 39, and 59 (Arg $\alpha\text{A}2$, $\beta\text{B}5$, $\beta\text{B}7$, $\beta\text{D}6$) guanidinium resonances were observed, indicating the stability of the arginine–pTyr interactions over this pH range (5–8). The arginine resonances do, however, experience differential exchange broadening behavior as a function of pH (Fig. 7). The H_ϵ resonances of three arginines not involved in phosphopeptide binding (Arg 27, Arg 30, and Arg 96) disappear most rapidly, at pH values below 7, due to exchange with solvent, as does that of Arg 91 whose aliphatic group only is involved in peptide binding at the +4 to +6 positions of the phosphopeptide (Pascal et al., 1994). This exchange behavior indicates that any hydrogen-bonding interactions involving the H_ϵ of these four residues must be weak and/or transient.

In contrast, the H_ϵ resonances of Arg $\beta\text{B}5$ and Arg $\beta\text{B}7$ show no exchange broadening even at pH 8.0, indicating that they are bur-

Fig. 7. Arginine H ϵ exchange broadening behavior as a function of pH. The pH at which the intensity is 20% of maximum is compared to (A) the measured rate of H ϵ exchange with solvent and (B) the $^1\text{H}-^{15}\text{N}_\epsilon$ order parameter (both sets of data from Pascal et al., 1995). Note that for two residues, Arg 37 and Arg 39, the H ϵ peaks are not significantly broadened by pH 8.0 and these residues are arbitrarily plotted at a pH value greater than 8.0.

ied by the phosphopeptide and/or make strong hydrogen-bonding contact with the phosphopeptide. It has been shown that Arg β B7 is involved in hydrogen-bonding interactions both with free phosphate and with the pY1021 phosphate group due to a strong downfield shift of the H ϵ resonance in phosphate buffer relative to imidazole buffer (Pascal et al., 1995). Such a downfield shift was not observed for the Arg β B5 H ϵ resonance; in fact, a slightly upfield shift was observed. However, recent structure calculations have shown that the Arg β B5 H η protons are involved in hydrogen bonding with the phosphate group, and in making these contacts, the Arg β B5 H ϵ and N ϵ are completely buried from solvent.

The remaining three arginine H ϵ protons have exchange behavior between these extreme cases. The H ϵ resonance of Arg β D6 broadens significantly by pH 6.7. The aliphatic side-chain positions of Arg β D6 contact the phenyl ring of the pTyr, but the guanidinium group is not likely to be involved based on results of ^{15}N relaxation experiments probing ps-ns motion of the $^1\text{H}-^{15}\text{N}_\epsilon$ bond vectors (Pascal et al., 1995). The H ϵ resonance of Arg 50 broadens significantly by pH 7.1. Based on the $^1\text{H}-^{15}\text{N}_\epsilon$ order parameter and the rate of exchange, Arg 50 may be involved in hydrogen-bonding interactions with carbonyl groups in the AB loop. Arg 27 and Arg 30 are found within this AB loop, but as discussed previously are surface exposed and are probably not involved in any intramolecular hydrogen-bonding interactions. Finally, the H ϵ resonance of Arg α A2 broadens to 20% of its maximum intensity by pH 8.0. Data from previous exchange and

relaxation experiments did not indicate a clear difference between the H ϵ resonance of Arg α A2 and Arg β B7 in terms of protection from exchange (Fig. 7; Pascal et al., 1995). Relaxation measurements, which monitor motion of the guanidinium group and cannot distinguish between stabilizing interactions formed by hydrogen bonds involving the H ϵ and H η protons, in fact showed a slightly higher order parameter for the $^1\text{H}-^{15}\text{N}_\epsilon$ of Arg α A2 than Arg β B7. However, chemical shift arguments are supportive of Arg β B7 H ϵ being involved in stronger hydrogen-bonding interactions than the Arg α A2 H ϵ , in that a much larger chemical shift change for the Arg β B7 H ϵ resonance occurs upon phosphate binding. Although the time scales of motion that influence ^{15}N relaxation and solvent exchange can be significantly different, the correlation between the $^1\text{H}-^{15}\text{N}_\epsilon$ order parameter and the exchange behavior (Fig. 7b) is intriguing and some insights may be gained by their comparison.

Data for the arginine H η protons are not shown, as most of these resonances are highly overlapped, and those that are distinct are very broad due to conformational exchange involving hindered rotation about the N ϵ -C ζ and C ζ -N η bonds. The majority of the H η resonances disappear due to exchange broadening by pH 7.0, with the exception of the two Arg β B7 H η resonances, which are protected from exchange until pH 7.2, and the Arg β B5 H η resonances. Although only two of the four potential H η resonances of Arg β B7 are observed, likely due to rapid rotation about the C ζ -N η bonds, Arg β B5 is unique in the PLCC SH2 domain-pY1021 complex in that all four H η resonances are observed (Yamazaki et al., 1995). These four resonances can be stereoassigned by considering intraresidue distances between the H ϵ and H η protons in arginine guanidinium groups (Pascal et al., 1997). The Arg β B5 H η_{11} and H η_{12} resonances are very weak and broad, but there is no pH dependence of the intensities. The resonances of H η_{21} and H η_{22} are sharper and have strong intensities throughout the titration, with no significant broadening by pH 8.0. These results further confirm the presence of stable hydrogen-bonding interactions involving the Arg β B5 guanidinium group H ϵ and H η protons.

In addition to arginine NH and NH $_2$ guanidinium resonances, a number of amide backbone resonances broaden and disappear over the course of the titration. These resonances are from residues in the disordered N-terminal and C-terminal portions of the SH2 domain (Gly 4, Ile 5, Ser 8, Glu 10, Glu 101, and Glu 102) or from loops (Asn 40 and Asn 43 in the BC loop, Gly 64 and Gln 65 in the D'E loop, Gly 70 and Asn 71 in the EF loop, Leu 77 near the FB loop and Arg 91 in the BG loop). Both the N- and C-terminal residues as well as the BC, D'E, and EF loops are poorly defined due to a lack of NOE restraints in structure calculations of the PLCC SH2 domain-pY1021 complex (Pascal et al., 1994). Some of these residues are in regions where backbone ^{15}N relaxation measurements show low $^1\text{H}-^{15}\text{N}$ order parameters (Farrow et al., 1994), i.e., the BC loop and N- and C-terminal residues, confirming greater mobility. Leu 77, which is completely exchange broadened prior to pH 8.0, has a high order parameter of 0.85 but a very large R_{ex} component indicating the presence of millisecond time scale motions. Thus, the exchange data is correlated with absence of hydrogen bonding, surface exposure, and conformational mobility either on the ps-ns time scale, giving rise to low-order parameters in NMR relaxation measurements, or on the millisecond time scale.

In summary, the pH titration behavior of residues of the PLCC SH2 domain-pY1021 complex has been investigated. In this complex, the pTyr of the pY1021 peptide and His β D4 residue of the PLCC SH2 domain have anomalously low pK_a values (<5.0). Stabilization of the -2 state of the phosphate of the pTyr in the

highly positively charged binding pocket is consistent with the theory of a strong electrostatic attraction leading to extremely rapid on-rates for SH2 domain interactions. Burial of His β D4 and stabilization of the N $_{\epsilon}$ -protonated tautomer through a hydrogen-bonding network similar to that observed in the crystal structures of other SH2 domain-phosphopeptide complexes leads to a lowering of the pK $_a$ value in both the peptide-complexed and free SH2 domains. This result provides additional insights into the role of the highly conserved His β D4 residue in positioning a critical arginine to allow pTyr-SH2 domain binding. His 87 as well as Glu 85 yield pK $_a$ values elevated from the free amino acid pK $_a$ by approximately 1 pH unit due to interaction with the helix dipole at the C-terminus of helix α B. These titration experiments also provide information concerning the exchange with solvent of NH and NH $_2$ protons of both backbone and arginine side-chains as a function of pH. Most arginine resonances broaden beyond detection due to exchange by neutral pH; however, the H $_{\epsilon}$ and the H $_{\eta}$ protons of Arg β B5 and the H $_{\epsilon}$ proton of Arg β B7 are not significantly broadened during the course of the titration. The H $_{\epsilon}$ proton of Arg α A2 did broaden but not significantly until pH 8.0, consistent with weaker hydrogen-bonding interactions involving this residue than those of Arg β B5 and Arg β B7.

Materials and methods

Cloning and purification of the C-terminal SH2 domain of bovine phospholipase C- γ 1 were performed as described previously (Pascal et al., 1994). The phosphopeptide, representing amino acids 1018–1029 of the β platelet-derived growth factor receptor (Asp-Asn-Asp-pTyr-Ile-Ile-Pro-Leu-Pro-Asp-Pro-Lys), was synthesized in the lab of Steve Shoelson. Numbering of the residues in the protein used in this paper is from the beginning of the recombinant construct as described previously (Pascal et al., 1994).

Titration experiments were performed over a pH range of 5 to 8, over which ionization states of histidine, cysteine, and phosphotyrosine can be probed. Resonances involved in phosphopeptide recognition were somewhat broadened at pH 5.0, indicating weaker binding; therefore, spectra were not recorded below pH 5.0 to avoid complications of contributions from the free SH2 domain to the measured pK $_a$ values. All NMR experiments were recorded on a Varian UNITY 500 MHz spectrometer equipped with a triple-resonance, pulsed-field gradient probe with an actively shielded z-gradient and a gradient amplifier unit, with the exception of ^{31}P spectra, which were collected on a Varian UNITY 500 MHz spectrometer equipped with an inverse-detected heteronuclear broadband probe. Spectra were processed with the NMRPipe software system (Delaglio et al., 1995) and analyzed with the program PIPP (Garrett et al., 1991).

pK $_a$ values for histidine residues were determined using ^1H - ^{15}N HMBC spectra (Pelton et al., 1993). These spectra were recorded on a 1 mM ^{15}N -labeled PLCC SH2 domain-pY1021 sample in buffer containing 50 mM sodium acetate, 50 mM sodium arsenate, 400 mM per-deuterated DTT and 40 mM EDTA. This sample was prepared by reducing the protein-peptide complex with 5 mM ^2H -DTT at 30°C for 1 h at pH 8.2 and then exchanging the sample into a buffer containing 50 mM sodium acetate, 50 mM sodium arsenate pH 5.0. Small amounts of 100 mM NaOH and HCl were used to vary the pH from 5.0 to 8.0 in 0.2–0.3 pH increments. The pK $_a$ values of the histidines of the free PLCC SH2 domain in the absence of phosphopeptide and phosphate were determined using a 0.2 mM sample that had been prepared by reduction of the

protein and exchange into a buffer containing argon-purged 100 mM sodium acetate, pH 5.0. The pulse sequence used for recording these HMBC experiments (Pelton et al., 1993) was modified by addition of z gradients but without sensitivity enhancement in a manner similar to that used for an experiment used to measure slow exchange processes in HSQC spectra (Farrow et al., 1994). Parameters for this experiment include a ^{15}N sweep width of 98.8 ppm centered at 205 ppm, a delay of 0.011 ms to obtain antiphase ^{15}N - ^1H magnetization (Pelton et al., 1993), 96–128 complex t1 points and 64–160 scans.

Gradient-enhanced ^1H - ^{15}N HSQC (Zhang et al., 1994) spectra were recorded on the same samples. These experiments were useful for monitoring binding at various pH values, for confirming the values of the pK $_a$ s obtained from measurement of histidine $^{15}\text{N}_{\delta 1}$ and $^{15}\text{N}_{\epsilon 2}$ chemical shifts, and for investigation of cysteine and acidic residue pK $_a$ values. Parameters for these experiments include 16–48 scans, 128 complex t1 points, and a sweep width of 78.9 ppm centered at 100 ppm to include arginine N $_{\epsilon}$ and N $_{\eta}$ correlations.

^{31}P experiments were recorded on the same samples to determine the pK $_a$ of the pTyr phosphate group in the PLCC SH2 domain-pY1021 complex. Experimental parameters include a sweep width of 19.8 ppm, 8,000 complex points and 14,000–20,000 scans per experiment. For measurement of the pK $_a$ of the free phosphopeptide (MW 1.45 kDa), a 5 mM sample was prepared in 50 mM sodium acetate, 50 mM sodium arsenate, 0.1 mM EDTA, pH 5.0. Small amounts of 100 mM NaOH and HCl were used to vary the pH from 4.5 to 7.8 in 0.2–0.3 pH increments. Each ^{31}P spectrum was recorded with 128 transients, an acquisition time of 1.0 s, and a dwell time of 2.0 s.

Chemical shift data were recorded as a function of pH and fit to the sigmoidal curve

$$y = [\delta_{\text{th}} + \delta_{\text{x}} * 10^{(\text{pH} - \text{pK}_a)}] / [1 + 10^{(\text{pH} - \text{pK}_a)}],$$

where y is the observed chemical shift and δ_{x} and δ_{th} represent the chemical shifts of the unprotonated and protonated species, respectively. Curve fits were initially performed using the program CRVFIT (Boyko & Sykes, 1993). A Monte Carlo routine provided by Dr. Neil Farrow was then used for error analysis, incorporating the uncertainties in measured chemical shifts and pH values.

Analyses of a number of SH2 domain structures were performed to compare hydrogen-bonding patterns involving His β D4. These included a refined solution structure of the PLCC SH2 domain-pY1021 complex (Pascal et al., 1997) based on the previously published structure (PDB filecode 2PLD; Pascal et al., 1994), as well as crystal structures of the N-terminal SH2 domain of Syp phosphatase (Lee et al., 1994), free (PDB filecode 1AYD), and in complex with phosphopeptides (PDB filecodes 1AYA, 1AYB, and 1AYC), the SH2 domain of Grb2 (PDB filecode 1GRI; Maignan et al., 1995), the Lck SH2 domain-phosphopeptide complex (PDB filecode 1LCJ; Eck et al., 1993) and the Src SH2 domain (Waksman et al., 1993), free (PDB filecode 1SPR), and phosphopeptide-complexed (PDB filecode 1SPS).

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