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Insights into the allosteric regulation of Syk association with receptor ITAM, a multi-state equilibrium †

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† Electronic supplementary information (ESI) available: the mathematical descriptions and fitting procedures of models, error analysis, simulated changes of concentration of each species of each protein/ligand system.

ABSTRACT

The phosphorylation of interdomain A (IA), a linker region between tandem SH2 domains of Syk tyrosine kinase, regulates the binding affinity for association of Syk with doubly-phosphorylated ITAM regions of the B cell receptor. The mechanism of this allosteric regulation has been suggested to be a switch from the high-affinity bifunctional binding, mediated through both SH2 domains binding two phosphotyrosine residues of ITAM, to a substantially lower-affinity binding of only one SH2 domain. IA phosphorylation triggers the switch by inducing disorder in IA and weakening the SH2-SH2 interaction. The postulated switch to a single-SH2-domain binding mode is examined using NMR to monitor site-specific binding to each SH2 domain of Syk variants engineered to have IA regions that differ in conformational flexibility. The combined analysis of titration curves and NMR line-shapes provides sufficient information to determine the energetics of inter-molecular binding at each SH2 site along with an intra-molecular binding or isomerization step. A less favorable isomerization equilibrium associated with the changes in the SH2-SH2 conformational ensemble and IA flexibility accounts for the inhibition of Syk association with membrane ITAM regions when IA is phosphorylated, and refutes the proposed switch to single-SH2-domain binding. Syk localizes in the cell through its SH2 interactions, and this basis for allosteric regulation of ITAM association proposes for the first time a phosphorylation-dependent model to regulate Syk binding to alternate receptors and other signaling proteins that differ either in the number of residues separating ITAM phosphotyrosines or by having only one phosphotyrosine, a half ITAM.

INTRODUCTION

The non-receptor spleen tyrosine kinase (Syk) is a central regulatory protein mediating signal transduction from immunoreceptors to various downstream targets¹ and is widely expressed in haematopoietic cells (B cells, mast cells, neutrophils and macrophages). Syk activity is closely related with many vital cellular events including cell proliferation, differentiation, polarization, cell-cell adhesion and phagocytosis.² Given its primary role in signaling, it is clear why dysfunction of Syk has been implicated in numerous inflammatory and autoimmune disorders (e.g. allergy, asthma, allergic rhinitis, atopic dermatitis, immune thrombocytopenia purpura, autoimmune hemolytic anemia, rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus).³ Abnormal expression of Syk is also associated with human B-cell malignancies and cancers (such as breast cancers, gastric cancers and melanomas),³⁻⁵ although the role of Syk in tumor progression is poorly understood; Syk appears to suppress tumor formation early in tumor genesis while being a promoter of tumor progression at later stages.⁶ The central function in numerous signaling pathways and phosphorylation of multiple protein substrates demands tight regulation of Syk activity. A complete characterization and understanding of the regulatory mechanisms of Syk activity is therefore particularly important given the disease relevance of Syk.

Inflammatory responses initiated by extracellular ligands binding membrane receptors lead to phosphorylation of cytosolic regions of the receptor known as immunoreceptor tyrosine-based activation motifs, ITAMs,^{7,8} and recruitment of Syk to the membrane. ITAMs contain two YXX(I/L) cassettes separated by 6-10 amino acids.⁹ Both tyrosine residues of ITAM need to be phosphorylated for optimal signaling¹. In B cells, the Src-family kinase Lyn phosphorylates the N-terminal YXX(I/L) cassette of the B-cell receptor but not the C-terminal one,^{1,10} which is likely phosphorylated by Syk itself¹. Doubly phosphorylated ITAM, dp-ITAM, thus serves as a docking site for Syk via its two tandem SH2 domains. The association of Syk with receptor dp-ITAM is high-affinity (nM),¹¹⁻¹⁶ and negatively regulated by tyrosine phosphorylation of Syk at position 130 (murine numbering), which lowers affinity, releasing Syk from the receptor.

The structural basis for a high-affinity interaction of Syk with dp-ITAM is readily explained by the crystallographic structure. Syk tyrosine kinase comprises two SH2 domains, (N)SH2 and (C)SH2 connected by interdomain A (IA), followed by interdomain B (IB) and a catalytic domain (Fig. 1a). The two SH2 domains together with IA are known as tandem SH2 domains (tSH2) and have a high sequence identity between human and murine Syk (92% for (N)SH2, 94% for (C)SH2, and 100% for IA). In the crystal form of the tSH2 fragment in complex with a dp-ITAM peptide, the SH2 domains are oriented with the two pY pockets optimally positioned to fit the spacing of the pYXX(I/L) cassettes of dp-ITAM in a head-to-tail fashion¹⁷ (Fig. 1b). This optimal spacing of the phosphotyrosine pockets exists in the six molecules in the asymmetric unit of the crystal structure (PDB ID 1A81), which differ in relative orientation of the two SH2 domains by $<18^\circ$. High-affinity binding thus derives from the large contact surface and combined association of tSH2 with two pYXX(I/L) cassettes. Interestingly, we note previously reported NMR residual dipolar coupling data established that in solution the SH2 domain-domain orientation of the unbound tSH2 resembles the orientation in the crystal structure of the dp-ITAM-bound tSH2, not unbound tSH2,¹⁸ which differ in orientation by $\sim 50^\circ$. The unbound SH2-SH2 domain structure is similar in the crystal structure for tSH2¹⁹ and full-length Syk,²⁰ so that neither have SH2-SH2 orientations consistent with the NMR residual dipolar couplings.

The regulatory tyrosine, Y130 (murine numbering; Y131 in human), lies in IA, distant to

the phosphotyrosine binding pockets of the SH2 domains (Fig. 1b). The effect of phosphorylation of Y130 in IA is to dissociate Syk from ITAM receptors, freeing Syk to engage in cytosolic or nuclear signal transduction pathways.²¹⁻²³ The introduction of negative charge at position 130 in Syk IA reduces the binding affinity between Syk tSH2 and dp-ITAM peptides by 2-3 orders of magnitude, and in the range of binding affinities that correspond to a single SH2 domain binding to phosphotyrosine peptides.^{13,14,18} Because Y130 is distal to the binding pockets of the SH2 domains (> 20 angstrom; see Fig. 1b) with no evidence for competition with the pYXX(I/L) cassettes for binding, this regulation is an allosteric control mechanism. It is distinguished from most other phosphorylation regulatory mechanisms in which tyrosine phosphorylation controls protein-protein interaction by direct effects of phosphorylation, either promoting binding by generation of the recognition determinant or inhibiting binding by steric conflict.²⁴

Earlier studies investigating the effects of Y130 phosphorylation on dynamics and conformation of Syk tandem SH2 showed that negative charge introduced into IA at position 130 causes large changes in NMR relaxation and sedimentation velocity, indicating that phosphorylation leads to a change in the conformational ensemble causing reorientation in the relative SH2 domain structure and a substantial weakening of the SH2-SH2 interaction.¹⁸ The conformational effect of phosphorylation is of considerably greater magnitude than the flexibility previously described for unphosphorylated Syk.¹⁶ It was suggested that the allosteric inhibition of the Syk-receptor interaction by IA phosphorylation was through a conformational change in domain structure that switched the bifunctional high-affinity binding across two SH2 domains to a monofunctional lower-affinity binding that involved only one SH2 domain.¹⁸ Thus, linker A phosphorylation was proposed to restrict the separation and relative orientation of two SH2 domains in a manner that no longer appropriately positioned the two phosphotyrosine binding sites to allow bifunctional binding, and therefore binding is by a single pYXX(I/L) cassette and the affinity for ITAM is reduced to a value comparable to a single SH2 domain.

Here we explore the molecular basis of the allosteric mechanism of IA phosphorylation in regulating Syk association with receptor ITAMs by affecting domain-domain structure. The consequence of Y130 phosphorylation on the ensemble of SH2-SH2 domain conformations was monitored by NMR relaxation. In addition, we exploited the residue-level information of NMR chemical shift to decompose the contribution to binding from each SH2 domain using a multistate analysis of binding equilibrium. We find that even though negative charge in IA does not completely disorder IA to the extent that the SH2 domains resemble two domains connected by a flexible linker, the affinity of phosphorylated tSH2 for receptor dp-ITAM is not reduced to monofunctional binding of a single SH2 to one pYXX(I/L) cassette, contrary to previous expectation. A multistate-binding analysis of ITAM association with tSH2 that accounts for discrete binding to each SH2 domain is found an effective approach for defining how IA Y130 phosphorylation alters the equilibrium of receptor binding. Together based on the binding analysis involved multiple complexes, we conclude the dramatic decrease of the apparent binding affinity after Y130 phosphorylation is largely an effect on the intra-molecular binding step after the initial inter-molecular association of phosphorylated tSH2 with dp-ITAM.

RESULTS

Interdomain A phosphorylation partially, but not fully decouples the two SH2 domains

The binding affinity of dp-ITAM to Syk depends on the optimal separation and relative

orientation of the tandem SH2 domains observed in the crystallographic structure (Fig. 1) to enable bifunctional binding of two YXX(I/L) cassettes simultaneously across both SH2 domains. To probe how IA phosphorylation alters the conformational ensemble of domain structure, we determine the overall rotational correlation times of the individual SH2 domains from NMR heteronuclear relaxation to characterize the extent of interaction between the SH2 domains in tSH2, a 28-kDa construct of Syk comprising the two SH2 domains plus IA. Strong interactions between (N)SH2 and (C)SH2 would result in a tightly coupled structure, manifest by a longer correlation time consistent with the overall ‘rigid-body’ structure defined by crystallography, while weakened SH2-SH2 domain coupling would be reflected by a shorter correlation time.¹⁸ The question addressed here is whether the SH2-domain structure of Syk phosphorylated on Y130 is one in which the two SH2 domains are only partly decoupled because phosphorylated IA retains some structural order, as would be necessary to hold the two binding pockets at a distance too long for bifunctional binding. Alternatively, are the SH2 domains fully decoupled due to a randomly disordered IA linker enabling independent rotational motion of the two SH2 domains, as was suggested from an alternative computational analysis²⁵ of the correlation times that opposed the original interpretation?¹⁸ NMR relaxation rates are compared for three proteins: unphosphorylated tSH2, tSH2_{PM}, which is the same construct but with the single amino-acid substitution Y130E to mimic phosphorylation, and tSH2_{FX}, which is a construct with IA residues 119-162 replaced by a 20-amino-acid flexible linker, (GGS)₃GS(GGS)₃. The Y130E substitution of tSH2_{PM} has been used in cells to mimic phosphorylation,^{21,26,27} and is reasonable given that Y130 is well solvated with few intra-molecular contacts consistent with the notion that phosphorylation serves to mediate the conformational ensemble of Syk domain structure rather than by a direct structural interaction. The (GGS)₃GS(GGS)₃ linker of tSH2_{FX} was shown to be flexible and enables independent rotation of the connected globular domains.²⁸

¹⁵N heteronuclear relaxation rates and *NOE* values measured at 800 MHz field strength for residues from tSH2, tSH2_{PM}, and tSH2_{FX} are shown in Fig. 2. Values are not shown for IA residues of tSH2 because these resonances are exchange broadened and unobservable, nor for IA resonances of tSH2_{FX} because of degeneracy. The *NOE* values from SH2 residues of all proteins are similar and near the long correlation-time limit of 0.83, while the *NOEs* for IA resonances of tSH2_{PM} are close to 0.5 reflecting greater internal mobility of this linker region. The long correlation-time limit occurs near 4 ns at 800 MHz and thus the SH2 domains are folded and well-ordered for the three proteins. In contrast, distinct residue-profiles for *T1*, *T2* and *T1/T2* values are observed for each of the three proteins. The relaxation-rate values for tSH2_{PM} are intermediate to those for tSH2 and tSH2_{FX}, and thus the hydrodynamic rotation of the SH2 domains of tSH2_{PM} is intermediate and not at the limit of tSH2_{FX} with a flexible linker between the two SH2 domains.

To quantify the hydrodynamic differences, rotational diffusion correlation times, τ_c , were obtained for tSH2, tSH2_{PM} and tSH2_{FX} by fitting the relaxation data for residues that have minimal internal motion and from an individual SH2 domain using the program TENSOR 2.0²⁹ (see Methods). The *T1/T2* values and estimated τ_c are summarized in Table 1. There is a good agreement between the *T1/T2* and τ_c values for (N)SH2 and (C)SH2 resonances. Similar to previous results¹⁸, τ_c estimated from experimental relaxation data of tSH2 is equal to 17 ± 1 ns for both SH2 domains, which is the same value predicted from the X-ray structure (17 ± 1 ns for the six molecules in the asymmetric unit of PDB ID 1A81) using the program HYRDONMR.³⁰ The dp-ITAM bound crystal structure of tSH2 is used to interpret the relaxation data rather than an unbound crystal structure in accord with Zhang, et al¹⁸ as noted above. Similar τ_c shows that the SH2 domain-domain interaction is strong and the two domains are tightly coupled and rotate

in solution as a rigid unit. For the other limit, tSH2_{FX} with a flexible linker has a much smaller τ_c equal to 8.2 ± 0.4 and 8.7 ± 0.4 ns determined from (N)SH2 and (C)SH2, respectively. These values are similar to 8.1 ± 0.4 ns obtained for N-SH2+IA, a construct comprising a single SH2 domain plus the linker residues for IA, so that the SH2 domains of tSH2_{FX} are found to rotate independently as expected for a flexible linker. By contrast, the τ_c value determined for tSH2_{PM} is 11 ± 1 ns for both SH2 domains, intermediate to tSH2 and tSH2_{FX}, suggesting that the linker of tSH2_{PM} is not as structurally ordered as in tSH2, but not fully disordered as in tSH2_{FX}.

In summary, phosphorylation increases conformational disorder of linker A causing the SH2 domains to rotate in solution like a partially coupled unit, as suggested previously by Zhang et al¹⁸ but contrary to an alternative interpretation by others²⁵ that the correlation times indicate the SH2 domains rotate independently as the beads-on-a-string model of polymers. Therefore the two SH2 domains of Syk phosphorylated in IA could be conformationally restricted such that the two pY binding pockets could not support bifunctional binding.

Characterizing the interaction of Syk tandem SH2 with ITAM by NMR

Given that the SH2 domains in tSH2_{PM} are not rotationally independent, it is reasonable that IA phosphorylation alters the domain conformational ensemble such that the SH2 domain separation is restricted to a distance too large to allow an ITAM molecule to associate bifunctionally. The reduced affinity of IA-phosphorylated Syk would therefore approximate the monofunctional affinity of a single SH2 domain. We therefore investigated the binding of Syk tandem SH2 proteins to peptides with one pYXX(I/L) cassette in comparison to a dp-ITAM peptide with two phosphotyrosines. The measurements reported here are for a dp-ITAM peptide derived from the CD3 ϵ chain of the T cell receptor, ITP, which has a reported affinity for Syk tSH2 of 20-30 nM.^{11,13} The two half peptides of ITP, each containing only one pY, are from the N-terminus, N-IHP, and the C-terminus, C-IHP. Peptide sequences are given in Fig. 1c.

The titration of tSH2 proteins with ITP, N-IHP and C-IHP was monitored by changes in ¹H-¹⁵N HSQC chemical shifts and line shapes. An advantage for investigating molecular interactions using NMR rather than other biophysical methods, such as gel filtration, equilibrium dialysis, calorimetry and fluorescence, is the residue-level information obtained compared to a single readout to follow binding. Of particular importance for the tSH2 system is that binding to a specific SH2 domain, (N)SH2 or (C)SH2, can be followed independently. Several resonances in each SH2 domain are well resolved in both the free and bound states and were used to monitor binding: G32, L37, L52, H61, Y73, A74, I99 and F106 for (N)SH2, and D175, G184, L192, C205, G210 and S244 for (C)SH2. The titration profiles within one SH2 domain were equivalent within experimental error and could be considered independent measurements of binding to that domain (see ESI).

NMR titration data were analyzed in the form of either binding curves or spectral line shapes,³¹⁻³⁴ as described in detail in ESI. Binding curves were generated from changes in the peak position for resonances in fast or fast-intermediate exchange on the NMR chemical-shift time scale based on obtaining hyperbolic binding curves as a function of free ligand concentration using fitted values of the equilibrium binding constants. In this exchange regime, the peak position approximates well the fraction of bound species, and the exchange rate is greater than approximately 2x the frequency difference between the free and bound states of the protein.³⁵ In contrast, the analysis of NMR line shapes utilizes the whole peak envelope and is not limited to resonances in the fast or fast-intermediate exchange regime. Line shape analysis was applied to all monitored resonances, including those in the slow-intermediate or slow regime

for cases of relatively higher binding affinity. Binding rate constants, as well as equilibrium binding constants were estimated from line-shape analysis.

Multi-site analysis for interactions of Syk tSH2 proteins with half ITAM peptides

The interactions between Syk tSH2 proteins (tSH2, tSH2_{PM}, or tSH2_{FX}) and the half ITAM peptides (N-IHP or C-IHP) were investigated to determine the single SH2-domain binding affinity. These monofunctional interactions were found to be low affinity, corresponding to the fast or fast-intermediate exchange regime, and thus both titration-curve and line-shape methods were used to extract the binding constants.

The NMR titration-curves and line-shapes were initially fit assuming a simple 2-state binding model: binding curves were fit using R³⁶ (home-written scripts utilizing DRC package³⁷) and line shapes were fit using LineShapeKin³⁵ (version 3.2, <http://lineshapekin.net>). Nevertheless, the best-fit 2-state model cannot explain the experimental data. The poor fit is illustrated with data from resonances of H61 and A74 in Fig. 3b and 3d, respectively. Data for H61 plotted as fraction bound vs. free [L] using the best-fit value for K_D show sigmoidal behavior and deviate systematically from the theoretical curve (Fig. 3b). Data for A74 illustrates the poor agreement between the experimental line shape and the predicted line shape (Fig. 3d). The poor fit of the NMR data indicate a model with more states is needed, which is reasonable given the protein has two potential binding sites, one on each SH2 domain, and the head-to-tail binding mode of Syk (N)SH2 and (C)SH2 binding to the C-terminal and N-terminal pYXX(I/L) cassette, respectively, is not absolute for the half ITAM peptides.

A 4-state model accounts for the data on the interactions between the tSH2 proteins and an IHP ligand. As shown in Fig. 4, the model contains four states for a tSH2 protein interacting with an IHP ligand: the unbound state, NC where N and C represent (N)SH2 and (C)SH2, respectively, the two states with one ligand bound to either SH2 domain, N^LC and NC^L, and the 2:1 complex with two ligands bound, N^LC^L. An IHP can bind to an SH2 domain with the equilibrium dissociation constant,

$$K_N = \frac{[NC][L]}{[N^L C]} = \frac{[NC^L][L]}{[N^L C^L]} \quad (1)$$

$$K_C = \frac{[NC][L]}{[NC^L]} = \frac{[N^L C][L]}{[N^L C^L]}$$

where L denotes an IHP ligand (N-IHP or C-IHP) with only one pYXX(I/L) cassette. To determine K_N and K_C , independent measurements for the IHP peptide association with (N)SH2 and (C)SH2 domains are needed and was possible with NMR by global fitting of peaks from the separate domains (see ESI for details). The improved fitting of the binding curves and line shapes obtained using the 4-state model is illustrated for resonances of H61 and A74 by comparing Fig. 4c with 4b and 4e with 4d.

Fitting results for the thermodynamic parameters of binding the IHPs to the three tSH2 proteins are summarized in Table 2. The dissociation constants K_N and K_C determined from fitting either the binding curves or the line shapes are in reasonable agreement. Values from the two methods are within a factor of 1.5 of each other, and do not vary systematically. It is observed that N-IHP or C-IHP bind a given SH2 domain in the three tSH2 proteins with similarly equivalent dissociation constants. Thus, the linker state does not affect the intrinsic binding

affinity of a single SH2 domain with the half ITAM, as expected.

The single SH2 domain affinities listed in Table 2 are unexpectedly low for the binding of a phosphotyrosine peptide to an SH2 domain. Other SH2 peptide complexes typically have dissociation constant values ranging from 20 nM to 50 μ M (ΔG^0 from -5.9 to -10.6 kcal/mol, SH2 domains from nine proteins).³⁸⁻⁴⁰ The affinity previously reported from an analytical ultracentrifugation method for Syk tSH2_{PM} binding dp-ITAM was 1.8 μ M,¹⁸ and therefore the notion that IA phosphorylation inhibits the Syk-receptor ITAM interaction by reducing the association to a single SH2 binding event is challenged by the significantly lower affinity of monofunctional SH2 binding determined here (Table 2).

Examination of Table 2 finds that (C)SH2 associates with N-IHP with an order of magnitude smaller dissociation constant ($K_C \sim 50-70 \mu$ M) than that of the other three half-ITAM complexes ($K_x \sim 300-800 \mu$ M). That (C)SH2 associates with N-IHP with higher affinity is of biological interest as it relates to the observation that a Src-family kinase phosphorylates the N-terminal tyrosine of ITAM only^{1,10} while Syk is thought to phosphorylate the C-terminal tyrosine.¹ Given the relative dissociation constants reported here, it follows that Src phosphorylation of the N-terminal tyrosine provides a docking site for Syk C(SH2), and so facilitates Syk in the subsequent phosphorylation of the C-terminal tyrosine of ITAM to generate the doubly phosphorylated ITAM for a stronger membrane anchoring of Syk to sustain membrane signaling.

With the fitted binding constants, the continuous change of concentration of each species in the 4-state model was simulated for each tSH2 construct binding with each IHP (see ESI). The three tSH2 constructs behaved similarly when binding to the same IHP because the single-domain binding affinities are similar as aforementioned. As expected, most tSH2 molecules form the two intermediate states (with only 1 IHP ligand bound; $N^L C$ and NC^L in Fig. 4) at the beginning of titration (the NC^L state has higher population due to its higher affinity, especially when binding with N-IHP); however, the concentration of these two intermediate states decreases as the concentration of ligand increases; Finally, the population of the 2:1 complex ($N^L C^L$ in Fig. 4) dominates at very high ligand concentrations.

Stepwise association of tSH2 proteins with full-length dp-ITAM

The domain-specific information provided by NMR enabled analysis of the effect the domain-structure ensemble and phosphorylation of IA on the energetics of dp-ITAM binding across two SH2 domains. NMR lineshape analysis was used to measure the association of the three Syk tandem SH2 proteins with the full-length ITP comprising two pYXX(I/L) cassettes. The IHP binding free energies from the results given in Table 2 indicate a complete binding scheme should account for the interaction of both ITP pYXX(I/L) cassettes with either SH2 domain and thus the simplest yet complete model includes ten states (Fig. 5a). The first binding step of the free tSH2 protein, NC, with ITP leads to one of four 1:1, monofunctional complexes with a single SH2 domain bound to one pYXX(I/L) cassette of ITP: $N^N C$, $N^C C$, NC^N and NC^C . (Superscript N and C refer to ligation by the N-terminal and C-terminal cassette of ITP, respectively.) For $N^C C$ and NC^N with head-to-tail association, the binding event for the second SH2 domain is either intra-molecular (isomerization) to form the bifunctional complex $N^C C'$, or inter-molecular to form a 2:1 ITP:tSH2 ternary complex. If the first binding event is not a head-to-tail association, then the intra-molecular event is assumed improbable and the second domain is occupied only upon binding a second ITP molecule. The four 2:1 ternary complexes after a second inter-molecular binding step are $N^N C^N$, $N^C C^N$, $N^N C^C$, and $N^C C^C$.

Each inter-molecular binding of an ITP pYXX(I/L) cassette with an SH2 domain is assumed to be independent. The equilibrium of (C)SH2 binding the N-terminal cassette of ITP is defined with the equilibrium dissociation constant K_{CN} ,

$$K_{CN} = \frac{[NC][L]}{[NC^N]} = \frac{[N^NC][L]}{[N^NC^N]} = \frac{[N^cC][L]}{[N^cC^N]} \quad (2)$$

where L denotes an ITP ligand which has two pYXX(I/L) cassettes. Analogous expressions define K_{NN} , K_{NC} , and K_{CC} . The equilibrium between tSH2 with ITP bound monofunctionally to a single SH2 domain and bound bifunctionally to both SH2 domains is described by the concentration-independent equilibrium isomerization constants, K'_N and K'_C ,

$$K'_N = \frac{[NC^N]}{[N^cC^N]} \quad (3)$$

$$K'_C = \frac{[N^cC]}{[N^cC^N]}$$

Based on the affinities determined for an SH2 domain to bind either N-IHP or C-IHP with a single pYXX(I/L) cassette (Table 2), the major species is NC^N after the first binding step, and N^cC' after the second binding step at sub-mM concentrations. Accordingly, it is worthwhile to consider a simpler 3-state model (Fig. 5b) along with the 10-state model. The first step of the reduced 3-state model is the (C)SH2 domain binding inter-molecularly with the N-terminal pYXX(I/L) cassette of ITP. The second step is the intra-molecular association (isomerization) of the (N)SH2 domain with the C-terminal pYXX(I/L) cassette of the same ITP molecule.

The NMR spectra for the titration of tSH2, tSH2_{PM} and tSH2_{FX} with ITP showed slow or intermediate exchange behavior as a result of higher binding affinities than N-IHP and C-IHP. An example of the slow-intermediate exchange behavior of resonances with larger chemical shift perturbation (CSP) and dual peak centers in the intermediate titration points is given in Fig. 6a. For peaks showing smaller CSP, a single peak was observed but the peak centers no longer represent the fraction bound (Fig. 6c); the position of the peak center plotted against ligand concentration displays a strong sigmoidal character due to intermediate exchange (Fig. 6e). Both types of residues were analyzed with the line-shape method (Fig. 6b & 6d).

The line-shape analysis for the tSH2 / ITP complexes was performed using both the 10-state and 3-state models (Fig. 5). A fit for all thermodynamic parameters is not possible, and thus the equilibrium dissociation constants of the inter-molecular binding steps were fixed to the values obtained from the line-shape analysis of the IHP complexes (Table 2), assuming the binding free energy of a single SH2 domain to the full-length ITP is approximately equal to that of IHPs. Fittings with either the 10-state or 3-state models gave equally good agreement with the experimental line shapes (Fig. 6b), and similar values for the equilibrium constants of the intra-molecular binding steps, K'_N and K'_C (Table 3). The 10-state model defines one more equilibrium parameter than the 3-state model: K'_C , the equilibrium dissociation constant for the intra-molecular binding between (C)SH2 domain and N-terminal pYXX(I/L) cassette. The off-rate values of the intra-molecular binding steps did not converge upon fitting and cannot be determined for the three ITP complexes due to lack of direct observable signal of the intermediate states. As might be expected, the intermediate states for tSH2 constructs binding with dp-ITAM do not have chemical shifts distinct from the final bound species based on the fact

that all of the resonances showed linear titration patterns.⁴¹ This inability to directly observe the intermediate state prevents determination of off-rate values.

Most relevant to the regulation of the Syk-receptor interaction is the estimate for K'_N , the equilibrium dissociation constant for the intra-molecular binding step of (N)SH2 to the C-terminal pYXX(I/L) cassette. The fitting of the 3-state model (Fig. 5b) yields K'_N values of $\ll 0.005$, $(38 \pm 8) \times 10^{-3}$, and $(120 \pm 20) \times 10^{-3}$ for tSH2, tSH2_{PM}, and tSH2_{FX}, respectively. The intra-molecular equilibrium (isomerization) favors bifunctional binding for all three forms of tSH2, even in the case of tSH2_{FX} with the flexible linker sequence between the two SH2 domains. For unphosphorylated tSH2 with the strongest SH2-SH2 interaction, the second intra-molecular binding step is too favorable to define K'_N accurately using line-shape analysis. Bifunctional binding is favored somewhat more strongly for tSH2_{PM} than tSH2_{FX} (2 to 3-fold stronger, $p < 0.05$ based on two-sided t -test).

The macroscopic apparent binding affinity based on the 3-state model ($K^{App} = K_{CN} \times K'_N$) is $\ll 0.3$, 3.2 ± 0.7 , and $6.1 \pm 1.3 \mu\text{M}$ for tSH2, tSH2_{PM}, and tSH2_{FX}, respectively. The apparent equilibrium dissociation constant of tSH2 and ITP is expected to be substantially lower than the limits achieved here. Reported values for the apparent K_d are ~ 0.001 to 30 nM from other methods,^{11–16} which is higher affinity than can be determined by direct NMR measurements.⁴¹

The concentration of each species over the course of the titration was simulated using fitted thermodynamic constants for the three tSH2 / ITP complexes (see ESI). For the 3-state model, concentrations of both the intermediate 1:1 monofunctional complex (NC^N) and the final 1:1 bifunctional complex (N'C') increase as the ligand concentration increases, and ratio of [NC^N]:[N'C'] remains the same (equals to K'_N) due to the first-order reaction nature of the intra-molecular binding step (isomerization). tSH2_{FX} / ITP showed higher concentration of the intermediate state than the other two due to its higher value of K'_N . The 3-state model of course is not accurate at high peptide concentrations. In comparison, the 10-state model behaved similarly as the 3-state model at ligand concentrations less than $\sim 450 \mu\text{M}$ (total protein concentration is $300 \mu\text{M}$); however, as the ligand concentration increases above $\sim 900 \mu\text{M}$, the formation of the 2:1 complex is favored, particularly for tSH2_{FX} / ITP due to the higher value of K'_N . These results of the 10-state model are more realistic than the 3-state model at high ligand concentrations, and may be required under certain physiological conditions as Syk was reported to form 2:1 complexes with the single-YxxL-containing receptors, CLEC-2.⁴²

CONCLUSION

The allosteric regulation of Syk binding to dp-ITAM regions of membrane receptors derives from the altered SH2-SH2 domain structure upon phosphorylation of the IA linker connecting the two SH2 domains. From a comparison of heteronuclear relaxation properties of tSH2, tSH2_{PM}, and tSH2_{FX}, the phosphorylation of IA at Y130 is found to weaken the SH2-SH2 interaction by inducing disorder in IA. Nonetheless, the SH2 domains are not rotationally independent to the extent of being joined by a fully flexible linker. The change in SH2-SH2 domain structure indicated by the reduction in τ_c cannot be accounted for by the differences among the six molecules in the asymmetric unit of dp-ITAM bound tSH2 (PDB ID 1A81) or the alternative SH2-SH2 domain structure in unbound full-length Syk (PDB ID 4FL2).

Site-specific free energies of binding determined from the residue-level information of NMR spectroscopy enable a description of the energetics for the separate binding to (N)SH2 and (C)SH2 domains that gives insight into the allosteric regulation of Syk by phosphorylation of IA. The change in the SH2-SH2 conformational ensemble upon introduction of negative charge at

position Y130 alters the binding process but does not restrict association to a single SH2 domain. Bifunctional binding was established for the association of the dp-ITAM peptide ITP with tSH2_{PM} negatively charged at position Y130 in IA, as well as tSH2_{FX} with a fully flexible linker. Given the flexibility of IA in tSH2_{PM} and tSH2_{FX}, which abolishes the stable domain structure matching the pYXX(I/L) spacing of ITAM, it was not apparent from the outset that tSH2_{PM} or tSH2_{FX} would engage in bifunctional binding in preference to association with two ITP ligands. The energetics of the site-specific association of each pYXX(I/L) cassette to separate SH2 domains (Table 2) establishes that for all three tSH2 proteins the binding free energy for bifunctional association of dp-ITAM is favored over forming the ternary complex with two inter-molecular binding steps (Table 3) at typical ligand concentrations (ligand:protein ratios less than 20:1; see ESI). The reduced affinity of Syk phosphorylated at Y130 for receptor ITAM is accounted for by a less favorable isomerization equilibrium to form the bifunctional complex N'C'. As a result, an important finding of this work is that IA phosphorylation alters the partitioning of the second association process between the bifunctionally associated ITAM-Syk complex and the ternary complex formed from two inter-molecular binding events. For tSH2_{PM}, the population of the ternary complex increases at concentrations that might approach those of clustered receptors (*i.e.* above 20:1). The low intrinsic affinity of a single Syk SH2 domain for phosphotyrosine-containing peptides is a key factor in the ability to regulate this partitioning; the affinity of a single SH2 domain binding must match the isomerization equilibrium to allow a switch from a lower-affinity bifunctional binding to a ternary complex populated at physiologically achievable receptor concentration. Regulation of this partitioning in a feasible concentration regime of clustered receptors would be important for controlling the competition of various membrane receptors or downstream signaling proteins that might associate with Syk through ITAMs with varying separation between pYXX(I/L), or importantly, receptors that have only a half ITAM with a single pYXX(I/L).⁴²

METHODS

Plasmid construction. cDNA encoding murine Syk tandem SH2 domains, Ser 8 to Gln 264, was cloned into a pET-30a(+) (Novagen) vector¹⁸ for the construct tSH2. The residue Y130 was mutated to glutamate using the QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene) for the construct tSH2_{PM}.¹⁸ The construct tSH2_{FX} has interdomain A (Phe 119 to His 162) substituted with a flexible 20-amino-acid linker [(GGS)₃GS(GGS)₃]. Two PCR reactions were performed for the tSH2_{FX} construct: the cDNA product of reaction 1 encodes (N)SH2 (Ser 8 to Pro 118) plus half of the linker (GGSGGSGGSG), and has cleavage sites for *NdeI* and *BamHI*; the cDNA product of reaction 2 encodes the other half of the linker (SGGSGGSGGS) plus (C)SH2 (Glu 163 to Gln 264), and has cleavage sites for *BamHI* and *XhoI*. These two pieces of cDNA were then cloned into the pET-30a(+) vector: the two inserts were cleaved by *NdeI*, *BamHI*, and *XhoI*; the vector was cleaved by *NdeI*, *EcoRV-HF*, and *XhoI*; ligation was performed using T4 DNA ligase. cDNAs for N-SH2+IA (Ser 8 to His 162) and N-SH2 (Ser 8 to Gly 117), with an N-terminal (His)₆ tag and TEV cleavage site, were cloned into pET-30a(+) vectors.

Protein expression and purification. Proteins were expressed in *Escherichia coli* strain Rosetta 2 (DE3) (Novagen). Cells were grown at 37 °C in M9 minimal medium containing ¹⁵N isotopes to an OD₆₀₀ reading of 1.0 to 1.3. Protein expression was induced by addition of 1 mM of IPTG and the cells were then kept at 18 °C for 18–20 h. Cells were collected by centrifugation and lysed using a French press. The lysate was subjected to ultracentrifugation and the soluble

fraction was collected. The proteins of tSH2 constructs were loaded onto a phosphotyrosine-agarose affinity column and eluted with a linear gradient (Buffer A: 50 mM Tris, pH 8.0; Buffer B: Buffer A plus 1 M NaCl). The proteins of N-SH2+IA and N-SH2 were purified with a HisTrap HP affinity column (GE Healthcare), cleaved with AcTEV protease (Invitrogen), and further purified with the HisTrap HP affinity column.

NMR sample preparation. The purified protein was concentrated to 0.8–1.0 mM with an Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and stored in buffer [50 mM sodium phosphate buffer, pH 7.5, 5 mM dithiothreitol (DTT), and 0.02% NaN₃] at 4 °C. The protein concentration of N-SH2 was determined by a Bradford assay kit (Thermo Scientific) due to the lack of Trp residues; the concentration of the other constructs was estimated from UV absorbance at 280 nm with extinction coefficients calculated by ExPASy ProtParam⁴³ (tSH2: 34380, tSH2_{PM}: 32890, tSH2_{FX}: 27390, N-SH2+IA: 17420 M⁻¹ cm⁻¹). The purity of samples was >95% based on SDS/PAGE analysis. The protein samples were 0.8–1.0 mM for relaxation experiments and ~0.3 mM for titration experiments.

The three peptides, ITP [Ac-PD(pY)EPIRKGQRDL(pY)SGLNQR-NH₂], N-IHP [Ac-PD(pY)EPIRKG-NH₂] and C-IHP [Ac-QRDL(pY)SGLNQR-NH₂], were purchased from EZBiolab. The purity of all peptides was >95% based on mass spectrometry. The peptides were dissolved in pure water (with pH adjusted to 7) and extensively dialyzed against pure water. The amount of peptide was estimated from UV absorbance at 267 nm with extinction coefficients from the literature¹³: 1304 M⁻¹ cm⁻¹ for ITP, 652 M⁻¹ cm⁻¹ for N-IHP and C-IHP. Samples were aliquoted, lyophilized, and stored at -20 °C before use.

NMR data collection. The NMR data were collected on a Bruker Avance-III-800 equipped with a 5mm TXI Z-gradient probe at 298 K. The T1, T2, and NOE ¹⁵N relaxation experiments were carried out using the TROSY-based pulse program TRT1ETF3GPSI, TRT2ETF3GPSI3D, and TRNOEF3GPSI.⁴⁴ Relaxation delays were 0.1, 0.1, 0.3, 0.5, 0.5, 0.7, 0.9, 0.9, 1.1, 1.3, 1.3, 1.5 s for T1 experiments, and 17.3, 17.3, 34.6, 51.8, 51.8, 69.1, 86.4, 86.4, 104, 121, 121, 138 ms for T2 experiments (the duplicated points were used to estimate uncertainties); the experiments were performed with these delays in a scrambled manner to minimize systematic error. In the ¹H-¹⁵N steady-state NOE experiment, a 3-s recycling time plus a 3-s proton saturation pulse were used for the NOE spectrum, while a 6-s recycling time was used for the reference spectrum. For titration experiments, a protein sample was sequentially mixed with the aliquoted and lyophilized peptide until >95% saturation, and the chemical shifts were monitored by 2D ¹⁵N-¹H TROSY HSQC experiments with 2048 points in the ¹H dimension and 256 points in the ¹⁵N dimension. All NMR experiments were performed at least two times.

NMR data analysis. The raw spectra were processed by NMRPipe⁴⁵ and visualized by SPARKY 3.113 (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA). Peaks of ligand-free proteins were assigned according to our previously published data.¹⁸ For peaks in the bound proteins, fast-exchange peaks were assigned by directly tracking the changes of chemical shifts while slow exchange peaks were assigned by TROSY-based 3D HNCA, HNCACB, HN(CO)CA, HN(CO)CACB experiments.

T1 and T2 values of individual peaks were extracted by fitting peak intensities $I(t)$ versus relaxation delays t to the exponential decay curve: $I(t) = I(0) * \exp(-t/T)$ (T : T1 or T2 value of the peak) using program Sparky2rate (J. P. Loria, Yale University) and Curvefit (A. G. Palmer, Columbia University). NOE values of individual peaks were calculated as the ratio of peak

intensities with and without proton saturation. To effectively compare the global motions, the interference of internal motions must be excluded; thus only residues which are within secondary structure elements,²⁹ with NOE values > 0.65 ,⁴⁶ and free from conformational exchange⁴⁶ were used for the summary and τ_c fitting. The rotational diffusion correlation time τ_c was fitted by TENSOR 2.0²⁹ using *TI*, *T2*, *NOE* values and the crystal structures of individual domains (PDB ID 1A81). The predicted correlation time for tSH2 and isolated SH2 domains was obtained by HYDRONMR³⁰ using the crystal structure (PDB ID 1A81, atomic element radius $a = 1.5 \text{ \AA}$, 298 K).

The model development and fitting procedures of NMR titration-curve and line-shape analyses are described in ESI.

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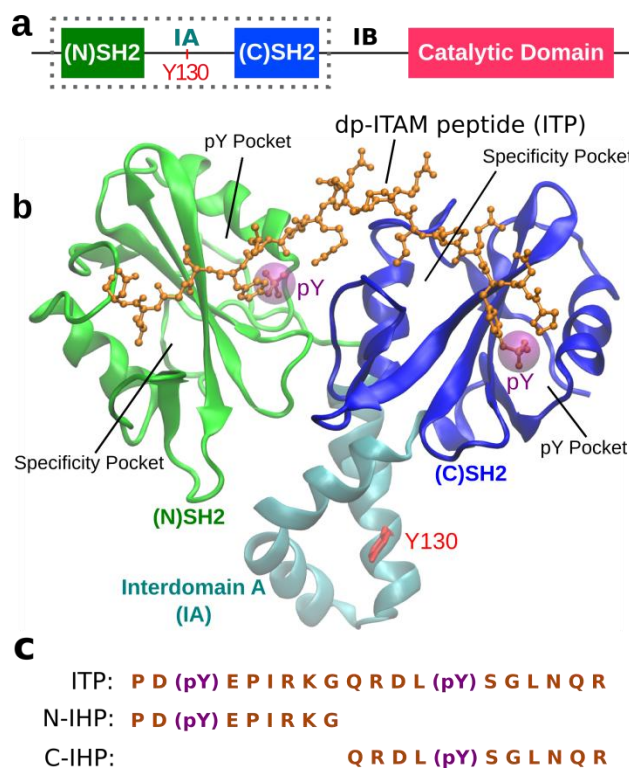


Fig. 1 The structure of Syk tyrosine kinase. **(a)** The domain structure of Syk: (N)SH2 domain (8-118), (C)SH2 domain (163-264), and catalytic domain (364-620) are connected by interdomain A (IA, 119-162) and interdomain B (IB, 265-363). The tandem SH2 domains (tSH2) (dotted box) contain (N)SH2, IA, and (C)SH2 (Residues are numbered according to murine Syk). **(b)** The ribbon representation of Syk tSH2 bound with a doubly phosphorylated ITAM (dp-ITAM) peptide (balls and sticks in orange) (PDB ID 1A81 chain A-B). The six molecules in the asymmetric unit of the crystal structure bind dp-ITAM peptide with the same spacing of pY and differ in relative orientation of the two SH2 domains by $<18^\circ$. Green: (N)SH2. Cyan: interdomain A. Blue: (C)SH2. Y130 in IA is shown with red sticks. The dp-ITAM peptide (ITP) is derived from the CD3 ϵ chain of the T cell receptor. The phosphoryl groups of the two phosphotyrosines (pYs) on ITP are shown as purple spheres. Binding is head-to-tail: (N)SH2 binds the C-terminal pY and (C)SH2 binds the N-terminal pY. **(c)** The sequence of ITP and the two half peptides, N-IHP and C-IHP.

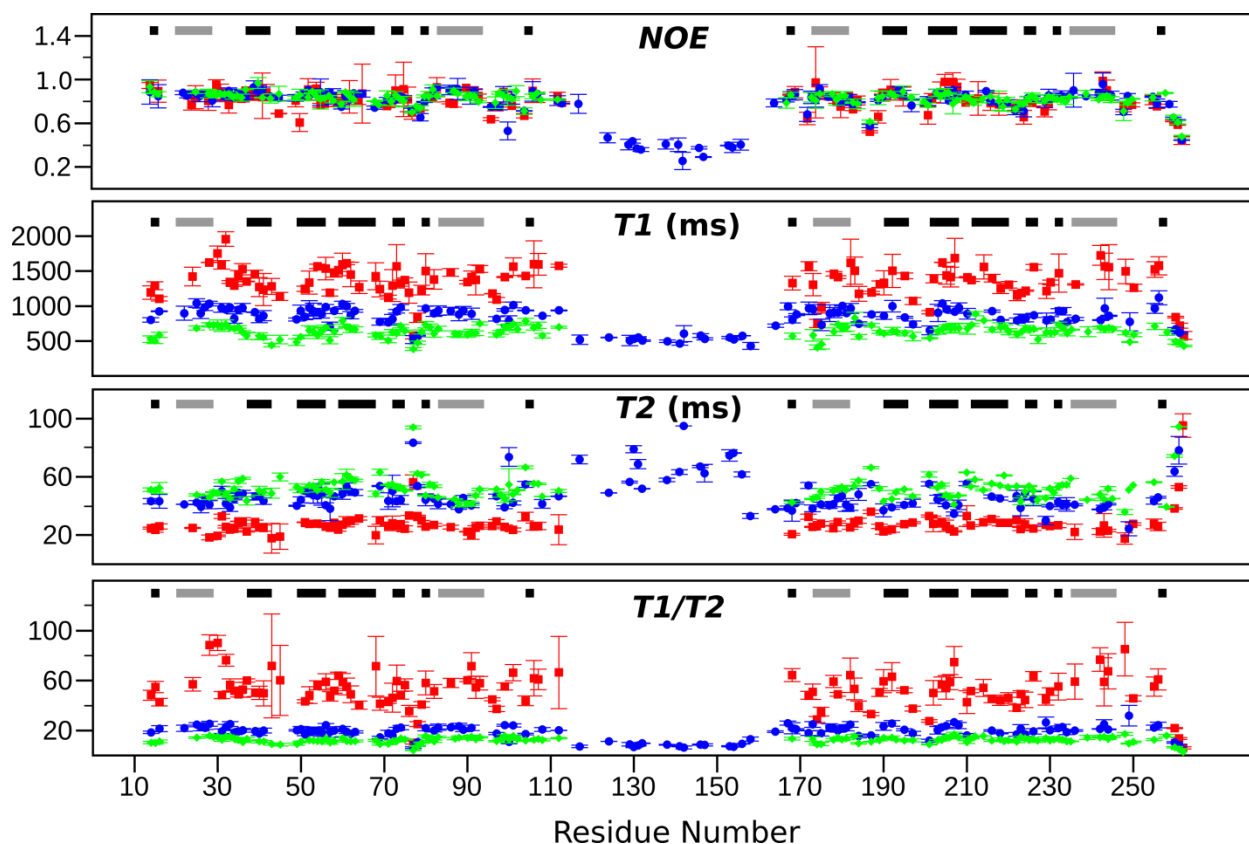


Fig. 2 Comparison of *NOE*, *T1*, *T2* and *T1/T2* values for Syk tSH2 constructs. Red: tSH2; Blue: tSH2_{PM}; Green: tSH2_{FX}. Data points represent the mean values and the error bars indicate standard deviations from two independent experiments. The bars on top denote secondary structure elements. Grey: α -helices. Black: β -sheets. The secondary structure elements are as follows: β A (residues 14–16, 167–169), β B (residues 37–43, 190–196), β C (residues 49–56, 201–208), β D β D' (residues 59–68, 211–220), β E (residues 72–75, 224–227), β F (residues 79–81, 231–233), β G (residues 104–106, 256–258), α A (residues 20–29, 173–182), α B (residues 83–94, 235–246). The resonances of IA are not observed in tSH2 and are degenerate in tSH2_{FX}.

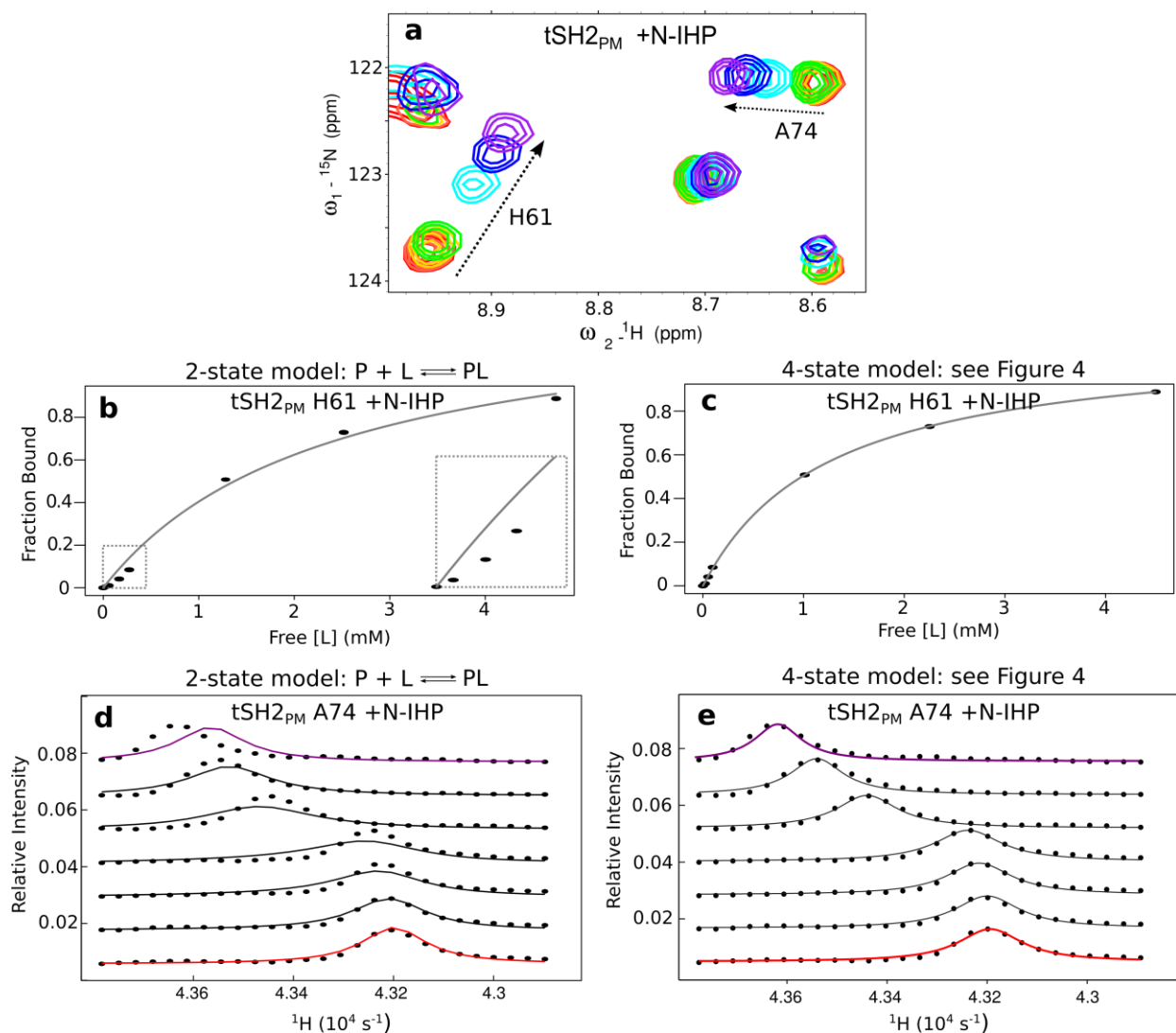


Fig. 3 Representative NMR ^{15}N -HSQC chemical shift titration data and comparison of fitting the 2-state model and 4-state model by titration-curve/line-shape analysis for IHP complexes. (a) Overlaid HSQC spectra for tSH2_{PM} (0.3 mM) titration with N-IHP. Increasing [N-IHP] corresponds to color changes from red, orange, yellow, green, cyan, blue, to purple. A zoomed region with H61 and A74 is shown as an example. The arrows indicate the shift of peaks during the titration process. (b-c) Titration-curve analysis for a fast-exchange residue, H61, in tSH2_{PM} plus N-IHP with either the conventional 2-state model or the 4-state model in Figure 4. The fraction bound values are the peak centers after normalization, and the free ligand concentrations are calculated with the indicated model. Dotted box: zoomed view. Circles: raw data. Line: predicted values with the indicated model. (d-e) Line-shape analysis for a fast exchange residue, A74, in tSH2_{PM} plus N-IHP with either the conventional 2-state model or the 4-state model. Circles: raw line-shape data. Lines: predicted line-shape with the indicated model (Red: the first titration point with no ligand. Purple: the last titration point with high concentration of ligand). The 4-state model fits well with the NMR data while the 2-state model does not.

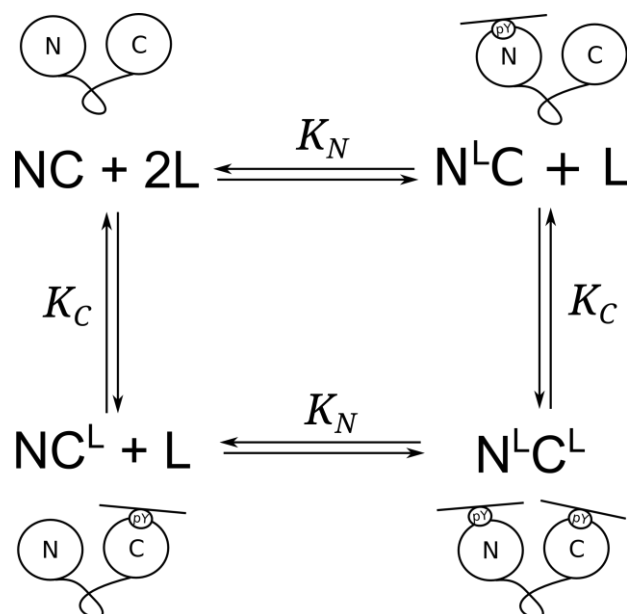


Fig. 4 The 4-state model for the interactions between a tSH2 protein and an IHP ligand with one pYXX(I/L) cassette. The possible tSH2 molecules are tSH2, tSH2_{PM}, or tSH2_{FX}. N and C represent (N)SH2 and (C)SH2, respectively. L denotes an IHP ligand with a single pYXX(I/L) cassette; the possible IHP ligands are N-IHP or C-IHP (see Fig. 1). The four states for the tSH2 molecule are the ligand-free state (NC), the two intermediate states with only one ligand bound to either the (N)SH2 domain (N^LC) or the (C)SH2 domain (NC^L), and the 2:1 complex which has a ligand bound to each of the two domains (N^LC^L). Assuming the two domains bind independently, then the system has only two dissociation constants, K_N and K_C for each domain, respectively.

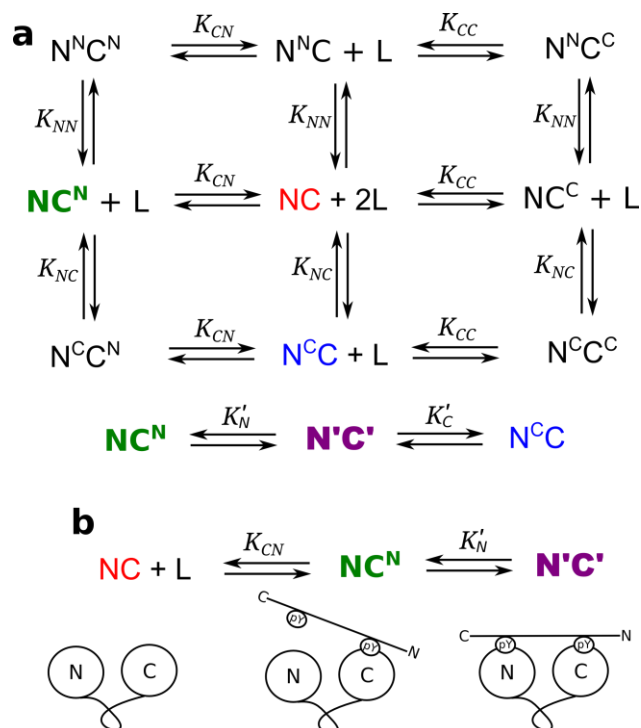


Fig.5 The 10-state and 3-state models for the interactions between a tSH2 molecule and the full-length ITP comprising two pYXX(I/L) cassettes (see Fig. 1). The possible tSH2 molecules are tSH2, tSH2_{PM}, or tSH2_{FX}. L denotes the full-length ITP with two pYXX(I/L) cassettes. **(a)** The 10-state model with all possible interactions between an SH2 domain (N or C) of tSH2 and the N- or C-terminal cassette (superscript N or C) of the full-length ITP. The first step is inter-molecular binding: a tSH2 molecule in the ligand-free state (NC, shown in red) binds either the N- or C-terminal cassette of ITP through either of its two domains to form a 1:1 monofunctional complex (N^NC, N^CC, NC^N, or NC^C). The second step can be another inter-molecular binding; the 1:1 monofunctional complex can bind to another ITP through its unbound domain to form a 2:1 ternary complex (N^NC^N, N^CC^N, N^NC^C, or N^CC^C). Alternatively, the second step can be an intra-molecular binding (isomerization) whereby N^CC or NC^N (shown in blue and green, respectively) form the 1:1 bifunctional complex N'C' (shown in purple; this is the head-to-tail binding complex; see Fig. 1). Assuming each SH2 domain binds an ITP peptide independently, the model has four equilibrium dissociation constants for the inter-molecular binding events: K_{NN} , K_{NC} , K_{CN} , and K_{CC} , where the first and second subscript indicates the SH2 domain and the ITP cassette, respectively. The model also has two equilibrium isomerization constants for the intra-molecular bindings: K'_N for NC^N isomerization to N'C', and K'_C for N^CC isomerization to N'C'. NC^N and N'C' (shown in bold) are predicted to be the major products of the first and second binding steps, respectively. **(b)** The simplified 3-state model. Considering only the major products of each step, the 10-state model can be simplified to this 3-state model. The structure of each state is shown below the name.

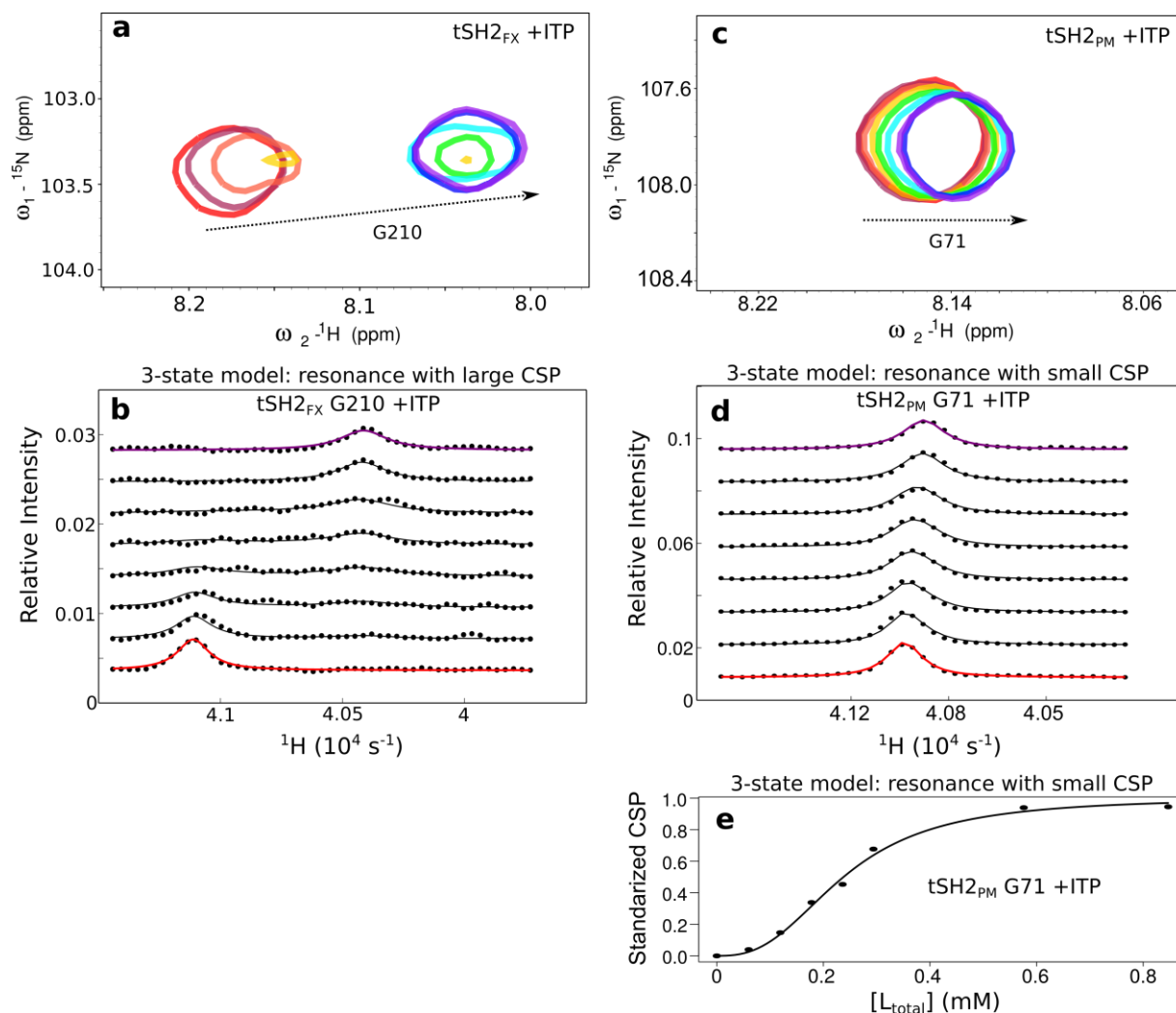


Fig.6 Representative NMR titration data and the line-shape analysis for ITP complexes showing the slower-exchange behavior than that of IHP complexes. **(a)** Overlaid HSQC spectra for tSH2_{FX} (0.3 mM) titration with ITP. Increasing [ITP] corresponds to color changes from red, orange, yellow, green, cyan, blue, to purple. A zoomed region with G210, which has relatively large CSP, is shown as an example. The arrow indicates the direction of change during the titration process. **(b)** Line-shape analysis for a slow-intermediate exchange residue, G210, in tSH2_{FX} plus ITP. Dotted lines: raw line-shape data. Solid lines: predicted line-shape with the 3-state model (Red: the first titration point with no ligand. Purple: the last titration point with high concentration of ligand). **(c)** Similar overlaid HSQC spectra for tSH2_{PM} (0.3 mM) titration with ITP showing G71, which has small CSP. **(d)** Line-shape analysis for G71 in tSH2_{PM} plus ITP with the 3-state model. Note that there is no significant line broadening due to the small CSP; however the apparent exchange rates are comparable to the difference in frequency so that exchange is in the intermediate regime. **(e)** Plot of the standardized CSP vs. [L_{total}] for the intermediate-exchange residue, G71, in tSH2_{PM} plus ITP. Circles: raw data. Line: predicted values with the 3-state model. Note the sigmoidal behavior due to intermediate exchange. Many residues in the ITP complexes showed this intermediate or slow-intermediate exchange behavior which prevents the titration-curve analysis because the shifts of peak centers no longer represent the fractions bound.

Table 1 Comparison of $T1/T2$, and correlation time (τ_c) for different protein constructs

Construct	Domain ^c	$T1/T2^d$	τ_c (ns) ^e	τ_{pred} (ns) ^f
tSH2	(N)SH2	53 ± 7	17 ± 1	17 ± 1 ^g
tSH2	(C)SH2	52 ± 10	17 ± 1	17 ± 1 ^g
tSH2 _{PM}	(N)SH2	21 ± 2	11 ± 1	-
tSH2 _{PM}	(C)SH2	22 ± 3	11 ± 1	-
tSH2 _{FX}	(N)SH2	12 ± 2	8.2 ± 0.4	-
tSH2 _{FX}	(C)SH2	13 ± 2	8.7 ± 0.4	-
N-SH2+IA ^a	-	13 ± 1	8.1 ± 0.4	-
N-SH2 ^b	-	6.4 ± 1.1	5.2 ± 0.3	5.9 ^h

^a Isolated N-terminal SH2 domain plus interdomain A, residues 8 to 162. ^b Isolated N-terminal SH2 domain. ^c Resonances from the individual domain in tSH2 used for averaging $T1/T2$ and calculation of τ_c . ^d Data are shown as mean ± SD of the qualified residues (> 20 residues) from each domain; values of the individual residues are means from two independent measurements. Only residues with low internal mobility and free from conformational exchange are used for this summary and τ_c calculation; see Methods for definition. ^e Correlation time computed using TENSOR 2.0 from experimental NMR measurements for each domain; data are shown as mean ± SD of the fitted values from two independent experiments. ^f Computationally predicted correlation time using HYDRONMR with the crystal structure (PDB ID 1A81, radius of the atomic elements $\alpha = 1.5$ Å, 298 K). ^g Mean ± SD of the predicted correlation time for the six molecules in the asymmetric unit of the crystal structure. ^h The predicted correlation time for the isolated C-terminal SH2 domain is 5.4 ns, similar to that of the isolated N-terminal SH2 domain.

Table 2 Dissociation and kinetic constants determined for individual SH2 domains of different IHP complexes with the 4-state model. The values for the SH2+IHP interaction relevant to the head-to-tail binding of dp-ITAM to tSH2 are shown in bold

Complex	(N)SH2				(C)SH2			
	K_N (μM) ^a	K_N (μM) ^b	k_{on}^N ($10^6 \text{ s}^{-1} \text{ M}^{-1}$) ^b	k_{off}^N (s^{-1}) ^b	K_C (μM) ^a	K_C (μM) ^b	k_{on}^C ($10^6 \text{ s}^{-1} \text{ M}^{-1}$) ^b	k_{off}^C (s^{-1}) ^b
tSH2 / N-IHP	450 ± 30	730 ± 20	2.5 ± 0.5	1800 ± 300	59 ± 6	69 ± 4	5.5 ± 0.4	380 ± 20
tSH2 _{PM} / N-IHP	820 ± 60	1300 ± 100	2.9 ± 0.3	3700 ± 300	69 ± 3	84 ± 4	6.6 ± 0.4	550 ± 20
tSH2 _{FX} / N-IHP	570 ± 20	820 ± 50	1.6 ± 0.3	1300 ± 200	50 ± 3	53 ± 7	6.6 ± 1.0	350 ± 20
tSH2 / C-IHP	510 ± 150	360 ± 40	2.6 ± 0.3	950 ± 60	350 ± 60	220 ± 40	12 ± 5	2600 ± 900
tSH2 _{PM} / C-IHP	540 ± 30	610 ± 110	3.3 ± 1.4	2000 ± 800	280 ± 20	310 ± 50	13 ± 11	4100 ± 3300
tSH2 _{FX} / C-IHP	570 ± 200	510 ± 90	3.9 ± 1.3	2000 ± 600	350 ± 130	290 ± 50	6.2 ± 3.3	1800 ± 900

^a Dissociation constants determined by titration-curve analysis. ^b Dissociation and kinetic constants determined by line-shape analysis. Data are shown as mean ± SD of fitted values from two independent experiments.

Table 3 Intra-molecular binding constants (see Fig. 5 for definition) and apparent equilibrium dissociation constants determined for ITP complexes of tSH2, tSH2_{PM} and tSH2_{FX}

Complex	3-state model		10-state model		
	$K'_N(10^{-3})$	$K^{App} (\mu\text{M})^a$	$K'_N(10^{-3})$	$K'_C(10^{-3})^b$	$K^{App} (\mu\text{M})^c$
tSH2 / ITP	$\ll 5$	$\ll 0.3$	$\ll 4$	$\ll 0.7$	$\ll 0.3$
tSH2 _{PM} / ITP	38 ± 8	3.2 ± 0.7	32 ± 10	4.4 ± 1.6	2.7 ± 0.8
tSH2 _{FX} / ITP	120 ± 20	6.1 ± 1.3	100 ± 10	11 ± 3	5.5 ± 1.1

$$^a K^{App} = K_{CN} \times K'_N.$$

$$^b K'_C = K_{CN} \times K'_N / K_{NC}.$$

$$^c K^{App} = K_{CN} \times K'_N = K_{NC} \times K'_C.$$

See Table 2 for the values of K_{CN} and K_{NC} determined from IHP complexes (line-shape analysis). Data are shown as mean \pm SD or detection limit of fitted values from three independent experiments.