Stereospecific gating of functional motions in Pin1

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Pin1 is a modular enzyme that accelerates the *cis-trans* isomerization of phosphorylated-Ser/Thr-Pro (pS/T-P) motifs found in numerous signaling proteins regulating cell growth and neuronal survival. We have used NMR to investigate the interaction of Pin1 with three related ligands that include a pS-P substrate peptide, and two pS-P substrate analogue inhibitors locked in the *cis* and *trans* conformations. Specifically, we compared the ligand binding modes and binding-induced changes in Pin1 side-chain flexibility. The *cis* and *trans* binding modes differ, and produce different mobility in Pin1. The *cis*-locked inhibitor and substrate produced a loss of side-chain flexibility along an internal conduit of conserved hydrophobic residues, connecting the domain interface with the isomerase active site. The *trans*-locked inhibitor produces a weaker conduit response. Thus, the conduit response is stereoselective. We further show interactions between the peptidyl-prolyl isomerase and Trp-Trp (WW) domains amplify the conduit response, and alter binding properties at the remote peptidyl-prolyl isomerase active site. These results suggest that specific input conformations can gate dynamic changes that support intraprotein communication. Such gating may help control the propagation of chemical signals by Pin1, and other modular signaling proteins.

allostery | protein dynamics | ligand dynamics | protein evolution

Phospho-serine/threonine-proline (pS/T-P) motifs are signaling motifs within intrinsically disordered loops of cell cycle proteins (1). The imide bond between the pS/T and P residues can adopt either the *cis* or *trans* conformation. These conformations differ in their susceptibility to kinases and phosphatases that propagate the chemical signals governing the cell cycle. Accordingly, the cell must regulate the *cis/trans* populations of these pS/T-P motifs to ensure proper signal routing.

In this context, the peptidyl-prolyl isomerase Pin1 has emerged as a critical regulator (2, 3). Pin1 is a reversible enzyme that catalyzes the *cis-trans* isomerization of the pS/T-P imide linkages (2, 3) of other signaling proteins, such as CDC25C, p53, c-Myc, NF-κB, cyclin D1, and tau (3). Pin1 engages when external events, such as S/T (de)-phosphorylation, change the *cis-trans* equilibrium. Pin1 then catalyzes the *cis-trans* isomerization, thereby accelerating the approach to the new equilibrium (1).

Pin1 is a modular protein of 163 residues consisting of a WW domain (1–39) and a larger peptidyl-prolyl isomerase (PPIase) domain (50–163) (Fig. 1). A flexible linker connects the two domains. Both domains are specific for pS/T-P motifs (1). The WW domain serves as a docking module, whereas catalysis is the sole province of the PPIase domain. Earlier structural studies of Pin1 revealed conformational changes upon substrate interaction, thus motivating flexibility-function studies of Pin1 (4–6). Our previous NMR deuterium relaxation studies of Pin1 mapped the changes in flexibility of methyl-bearing side chains caused by interaction with an established phospho-peptide (pT) substrate (6). The interaction caused both gains and losses in side-chain flexibility. The flexibility losses occurred along a conduit of conserved hydrophobic residues within the PPIase domain, linking the domain interface with the PPIase catalytic site. These sites are on opposing sides of the PPIase domain, separated by distance of approximately 12 Å (Fig. 1).

Fig. 1. The molecules of this study. (A) Surface representations of Pin1 [PDB ID code 1PIN (14)]; PPIase domain (aqua); WW domain (magenta); PPIase active site (yellow) with key residues labeled. (B) Pin1 methyls (spheres); methyls at functional regions (the domain interface, the PPIase active site, and the PPI catalytic loop) are colored according to domain (PPIase aqua; WW magenta). The gray spheres are methyls lacking a known function. (C) The three Pin1 ligands, left to right: substrate FFpSPR, *cis* locked inhibitor, and *trans* locked inhibitor.

Further interpretation of these results was hindered by the interconversion of the substrate pS/T-P imide bond between the *cis* and *trans* conformations. Pin1 recognizes both conformers. Thus, if and how the two conformers might impose different changes in dynamics remained indeterminate.

Etzkorn and coworkers designed peptidomimetic analogs of the Pin1 phospho-serine substrate (FFpSPR) (7, 8) (Fig. 1). These analogs replace the substrate pS-P core with alkene isomers that lock the imide as *cis* (Ac-Phe-Phe-pSer-Ψ[(Z)CH = C]-Pro-Arg-NH₂), or *trans* (Ac-Phe-Phe-pSer-Ψ[(E)CH = C]-Pro-Arg-NH₂). These ligands are competitive inhibitors of Pin1 PPIase activity ($K_{i,cis} = 1.7 \pm 0.1 \mu\text{M}$, and $K_{i,trans} = 40 \pm 2 \mu\text{M}$) (8), and are proxies of the substrate *cis* and *trans* ground states, thus providing a unique opportunity to distinguish changes caused by the *cis* versus *trans* conformers.

Here we describe NMR studies of the interaction of these locked inhibitors and their parent substrate, FFpSPR, with

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Pin1 (Fig. 1C). The *cis* and *trans* inhibitors displayed different binding modes that imposed different changes in Pin1 side-chain flexibility. The *cis* inhibitor caused more prominent conduit rigidity than the *trans* inhibitor. Interactions between the PPIase and WW domains enhanced the conduit response and altered the ligand binding properties at the distal PPIase active site. These results suggest that specific input conformations gate changes in Pin1 dynamics that support intraprotein communication. Such stereoselective gating may be another means by which flexible proteins regulate the propagation of chemical signals.

Results

Comparison of Ligand Binding Modes. To define the ligand binding modes, we measured amide ^{15}N - ^1H chemical shift perturbations (CSPs) in Pin1 caused by adding saturating amounts of ligand (Fig. 2 and *SI Text*). The substrate FFpSPR produced CSPs in both the PPIase and WW domains, with a strong bias toward the WW domain. This bias echoes other Pin1 substrates (4). By contrast, the CSPs of the locked inhibitors were heavily biased toward the PPIase domain, consistent with their competitive inhibition of PPIase activity (8). The *cis* inhibitor bound the isolated PPIase domain (40–163, *vide infra*) of Pin1 more tightly than the *trans*- (average $K_{\text{d,cis}}^{\text{PPI}} = 7 \mu\text{M}$, average $K_{\text{d,trans}}^{\text{PPI}} = 66 \mu\text{M}$) (Table S1).

The two inhibitors showed different domain binding preferences (Fig. 2). The *trans* inhibitor gave significant CSPs (>0.05 ppm) in both the WW and PPIase domain, with the latter perturbations being more pronounced. The K_{d} values were similar for both domains (average $K_{\text{d,trans}}^{\text{WW}} = 53 \mu\text{M}$, average $K_{\text{d,trans}}^{\text{PPI}} = 37 \mu\text{M}$). By contrast, the *cis* inhibitor CSPs resided almost exclusively within the PPIase domain (average $K_{\text{d,cis}}^{\text{PPI}} = 28 \mu\text{M}$); the only significant WW CSP was to H27 at the domain interface (Table S1). Thus, whereas *trans* inhibitor can bind either domain, the *cis* is a selective PPIase domain binder.

Within the PPIase domain, the *cis* and *trans* inhibitors gave distinct CSP signatures that we exploited in competition binding experiments. We initially saturated Pin1 with the *trans* inhibitor, and, as expected, we observed its CSP signature in both the PPIase and WW domains. Subsequent additions of the *cis* inhibitor gradually transformed the PPIase CSP signature from that of *trans* to *cis*, whereas the WW domain retained the *trans* signature (Fig. 3). The converse titration of *trans* into a preexisting solution of Pin1/*cis* produced the same results. These results show

that Pin1 can bind simultaneously the *cis* conformer in the PPI domain, and the *trans* conformer in the WW domain.

PPIase Activity Is Modulated by Remote Interactions at the Domain Interface. Previous structural studies (9) showed that pS/T phospho-peptide substrates had slightly different binding affinities for the isolated PPIase domain versus full-length Pin1, suggesting the modulation of PPIase binding affinity by interdomain interactions. The locked inhibitors invited further investigation of this effect, with the advantage that we could now interpret the results stereospecifically.

We therefore generated a truncated Pin1 construct (Pin1-PPI, 40–163) lacking the WW domain (residues 1–39). The ^{15}N - ^1H spectra of Pin1-PPI and full-length Pin1 were nearly superimposable. Deviations were at the interdomain interface (F134, S138, F139, A140, L141, R142, S147, and I159), as with earlier investigations (10). The WW domain thus acts as another ligand of the PPIase domain, with a binding surface defined, in part, by these interfacial residues. As in earlier studies, Pin1-PPI retained isomerase activity (5, 11, 12); however, it was higher than full-length Pin1 (13) by a factor of approximately 4.5. Similar behavior was observed with Pin1 substrates derived from the Tau protein (11).

We compared the inhibitor binding modes to Pin1-PPI versus full-length Pin1 by comparing the resulting ^{15}N - ^1H CSPs. The *cis* inhibitor gave nearly identical CSPs for Pin1-PPI and full-length Pin1. By contrast, the *trans* CSPs differed between Pin1-PPI and full-length Pin1 at the domain interface (F139 and A140), the catalytic loop (H64, S71), and the substrate proline binding pocket (R127, G128, and Q131).

Unexpectedly, the CSPs of H59, S67, R119, and Q129 revealed slightly higher affinity (two- to fourfold smaller K_{d} values) of the *cis* inhibitor for the truncated Pin1-PPI over full-length Pin1 (Table S1). Except for R119, all of these residues are part of PPIase active site (14). This affinity mismatch cannot be due to direct binding to the WW domain because the *cis* inhibitor is a selective PPIase domain binder (*vide supra*). Instead, the K_{d} s suggest that interdomain interactions at one site (the WW domain interaction surface of the PPIase domain) can alter binding properties at a remote site—the substrate pocket on the opposite side of the PPIase domain.

Binding-Induced Changes of Side-Chain Flexibility. We mapped the changes in Pin1 side-chain dynamics on the subnanosecond time scale caused by interactions with the substrate FFpSPR and

Fig. 2. NH CSPs of the three ligands. (A) Substrate FFpSPR; (B) *cis* locked inhibitor; and (C) *trans* locked inhibitor. Significant CSPs were those >0.05 ppm (horizontal dashed line). Except for H27, the significant *cis* CSPs (B) are just PPIase domain residues, whereas the *trans* CSPs (C) include residues of both domains.

Fig. 3. Competition of the *cis* and *trans* locked inhibitors for binding to the WW (1–39), linker (40–49), and PPIase (50–163) domains illustrated by amide ^1H line-shapes from ^{15}N - ^1H cross-peaks. (A rows): Pin1 saturated with the *cis* inhibitor (black traces and vertical blue solid lines) or the *trans* inhibitor (red traces and vertical dashed magenta lines). (B rows): Chemical shifts under simultaneous saturation of *cis* and *trans*: WW binds the *trans* inhibitor whereas the PPIase binds the *cis* inhibitor.

the two locked inhibitors. This involved measuring deuterium (^2D) longitudinal ($R_1(^2\text{D})$) and transverse ($R_{1\rho}(^2\text{D})$) relaxation rate constants for the CH_2D isotopomers of methyl-bearing side-chains in $\text{U-}^{15}\text{N}/^{13}\text{C}$, 50% ^2D Pin1 at two static field strengths, 16.4 and 18.8 T (example spectra in Fig. S1). Measurements of isolated (apo) Pin1 came from our previous studies (6).

The deuterium $R_1(^2\text{D})$ and $R_{1\rho}(^2\text{D})$ values report on the sub-nanosecond ($<10^{-9}$ s) motions of the methyl ^{13}C - ^2D bond relative to the external field $\mathbf{B}_0$ (15). We used the Lipari-Szabo formalism (16–18) to extract motional parameters from the $R_1(^2\text{D})$ and $R_{1\rho}(^2\text{D})$ values. The Lipari-Szabo parameters describe ^{13}C - ^2D bond motions due to conformational dynamics via site-specific order parameters S^2_{axis} , and effective correlation times, τ_e . S^2_{axis} is a pure number between 0 and 1 that measures the amplitude of the reorientational motions experienced by the methyl ^{13}C - ^{13}C symmetry axes. S^2_{axis} decreases with increasing motional amplitude. The correlation time τ_e estimates the rapidity of the motions underlying S^2_{axis} .

Determining S^2_{axis} and τ_e requires prior characterization of overall Pin1 tumbling. Hence, we measured backbone amide ^{15}N relaxation parameters ($R_1(^{15}\text{N})$, $R_2(^{15}\text{N})$) and steady-state ^{15}N - ^1H NOE, followed by reduced spectral density mapping (19–21). This gave for each amide nitrogen-hydrogen (NH) bond two model-independent parameters of reorientational motion, $J(0)$ and $J(\omega_{\text{N}})$; both are highly sensitive to overall tumbling. The $J(0)$ and $J(\omega_{\text{N}})$ values consistently partitioned into two clusters, corresponding to the WW and PPIase domains (Fig. S2). This clustering suggested an approximate approach of using domain-specific, isotropic correlation times for overall tumbling (*SI Text*). Thus, after excluding NHs with outlying $J(0)$ values due to substantial subnanosecond or μs -ms dynamics (Figs. S3 and S4) we determined $\tau_{m,\text{WW}}$ and $\tau_{m,\text{PPIase}}$ for each Pin1 state, using the corresponding $R_2(^{15}\text{N})/R_1(^{15}\text{N})$ ratios (22).

We then fit the side chain deuterium $R_1(^2\text{D})$ and $R_{1\rho}(^2\text{D})$ rates to the Lipari-Szabo spectral density function (*SI Text*, Eq. S7) using S^2_{axis} and τ_e as adjustable parameters. After excluding methyls suffering resonance overlap or poor signal-to-noise, we could fit a common set of 53 methyl groups for apo Pin1 and the three complexes (Tables S2 and S3). The majority of these methyls (43) are in the PPIase domain. The S^2_{axis} values ranged from 0.08 to 0.98, and had an average value and uncertainty of

0.77 ± 0.03 . The effective internal motion τ_e values ranged from 10 to 260 ps/rad, with an average value and uncertainty of 55 ± 4 ps/rad.

The formation of each Pin1-ligand complex caused changes in side-chain flexibility given by $\Delta S^2_{\text{axis}} = S^2_{\text{axis,COMPLEX}} - S^2_{\text{axis,APO}}$. Fig. 4 maps ΔS^2_{axis} onto the X-ray crystal structure Protein Data Bank (PDB) code 1PIN (14) (corresponding bar-graphs are in Fig. S5). Positive ΔS^2_{axis} indicates a loss of flexibility (red spheres), whereas negative ΔS^2_{axis} indicates a gain of flexibility (blue spheres). The magnitudes of the ΔS^2_{axis} were typically approximately 0.05–0.08, with the largest changes approximately ± 0.3 .

Fig. 4. Colored spheres showing ΔS^2_{axis} for the methyl-bearing side chains of Pin1 caused by binding (A) FFpSPR; (B) *cis* locked inhibitor; and (C) *trans* locked inhibitor. (D) The ΔS^2_{axis} for the truncated Pin1-PPI upon binding the *cis* locked inhibitor. The color code is red = $\Delta S^2_{\text{axis}} > 0$ (stiffening), blue = $\Delta S^2_{\text{axis}} < 0$ (loosening), white (no significant change). The color intensity scales with the magnitude of ΔS^2_{axis} .

To compare patterns of ΔS^2_{axis} across the Pin1–ligand interactions, we wanted a metric sensitive to: (i) prominent ΔS^2_{axis} changes; (ii) both positive and negative ΔS^2_{axis} (flexibility losses and gains). Thus, we used a correlation coefficient

$$r(i,j) = \sum_{k=1}^{N_{\text{METHYLS}}} \left\{ \frac{\Delta S^2_{i,\text{axis}-k} * \Delta S^2_{j,\text{axis}-k}}{C_i C_j} \right\}, \quad [1]$$

$$\text{where } C_{ij} = \sqrt{\sum_{k=1}^{N_{\text{METHYLS}}} (\Delta S^2_{ij,\text{axis}-k})^2},$$

where the “i” and “j” refer to distinct Pin1 ligands. The summation index “k” runs over the methyls common to the two Pin1 interaction complexes. Methyls with similar ΔS^2_{axis} , in both magnitude and sign, contribute positively to $r(i,j)$, and $r(i,j)$ values closer to 1.0 (the maximum value) indicate greater similarity. Uncertainties in $r(i,j)$ were estimated using the standard error in the correlation coefficient (23) (*SI Text*).

Substrate Binding Reproduces the Hydrophobic Conduit. Pin1 side-chain flexibility both increased ($\Delta S^2_{\text{axis}} < 0$) and decreased ($\Delta S^2_{\text{axis}} > 0$) upon interaction with the substrate FFpSPR. Decreases occurred mainly for PPIase methyls within or adjacent to the substrate active site (L61 δ_1 , I78 δ_1 , L122 δ_1 , A124 β , M130 ϵ , and I156 γ_2), and the domain interface (e.g., I28 δ_1 , I93 δ_1 , L141 δ_1/δ_2 , and V150 γ_1) (Fig. 4). These stiffening side chains formed an internal conduit, similar to that seen for Cdc25 (6). This was supported by $r(\text{FFpSPR}, \text{Cdc25}) = 0.66 \pm 0.08$. These similar changes in flexibility are reasonable, because both ligands are substrates. Yet, there were also local dissimilarities. Cdc25 caused greater stiffening of the conserved hydrophobic cluster, L60, L61, and V62 near the PPIase active site (FFpSPR showed significant stiffening only at L61), whereas FFpSPR showed greater stiffening in the substrate pocket (L122 δ_1 , A124 β , and M130 ϵ). Thus, the details of the conduit response may depend on whether the substrate is pT versus pS, and on the residues flanking the central pS/T-P core.

Inhibitor Binding Indicates Stereoselective changes in Flexibility. The *cis* locked inhibitor caused the most extensive loss of side-chain flexibility. By contrast, the *trans* locked inhibitor caused a more heterogeneous pattern of ΔS^2_{axis} , which included a greater number of flexibility increases (loosening). To better compare their dynamic effects, we correlated their effects with those of their parent substrate FFpSPR, and found $r(\text{FFpSPR}, \text{TRANS}) = 0.19 \pm 0.14$, whereas $r(\text{FFpSPR}, \text{CIS}) = 0.64 \pm 0.08$. These disproportionate r -values suggested that the *cis* inhibitor reproduced the substrate conduit response more prominently than the *trans* inhibitor (compare to Fig. 4). The Pin1-*trans* r -value is lower in part because it lacks contributions from active site methyls L122 δ_1/δ_2 ; they were nearly gone in the Pin1-*trans* deuterium relaxation spectra due to severe ^{13}C exchange broadening. Such broadening was absent for both Pin1-*cis* and Pin1-FFpSPR. Such differential broadening provides another example of the different dynamic responses provoked by the *cis* versus *trans* inhibitors, and of the greater dynamical similarity of Pin1-*cis* (over Pin1-*trans*) to Pin1-FFpSPR.

Further evidence for dynamical divergence between Pin1-*cis* and Pin1-*trans* came from the differences in their S^2_{axis} values (Fig. S6). Significant differences occurred at the proline binding pocket (L60 δ_1 , V62 γ_2 , M130 ϵ , I158 δ_1), the catalytic loop (I78 δ_1 , T79 γ_2), and the domain interface (I89 γ_2 , I93 δ_1 , V150 γ_1 , T152 γ_2 , T162 γ_2). Moreover, many of these same residues showed divergent *cis/trans* responses in other dynamical parameters including: (i) backbone NH $J(0)$ values (ii) $^{13}\text{C}_{\text{methyl}} R_1 * R_2$ values for Pin1-*cis* versus Pin1-*trans* (Fig. S6). Thus, multiple parameters

show that changes in Pin1 flexibility are sensitive to the *cis* versus *trans* configuration of the ligand.

Collectively, the FFpSPR substrate and corresponding *cis*- and *trans*-locked inhibitors demonstrated that: (i) the flexibility changes in the conduit—marked by stiffening of the more buried side chains—is an intrinsic response of Pin1 to substrate binding; (ii) the response is stereoselective—the *cis* conformer is the more powerful conduit stimulator.

WW Domain Binding Modulates the Conduit Response. The *cis* inhibitor has negligible interactions with the WW domain (vide supra). Yet, deleting the WW domain (yielding Pin1-PPI) changed the binding affinity of the *cis* inhibitor at the remote PPIase active site. This provoked our interest in the conduit response in the absence of the WW domain. We therefore mapped ΔS^2_{axis} for Pin1-PPI caused by binding of the *cis* locked inhibitor (Fig. 4). The ΔS^2_{axis} values were similar to that of full-length Pin1, albeit less prominent, with an overall correlation of $r(\text{CIS} : \text{Pin1}, \text{CIS} : \text{Pin1-PPI}) = 0.47 \pm 0.11$. Thus, whereas the conduit response is principally encoded within the PPIase domain, interdomain interactions can modulate its magnitude.

Dynamic Changes Conserved Across the Pin1-Ligand Complexes. To define more precisely the flexibility changes important for binding, we pinpointed the recurrent changes across four interactions including the three ligands herein, and our previous Cdc25 study (6). For example, ligand binding caused consistent loosening ($\Delta S^2_{\text{axis}} < 0$) at methyl axes of A53 β , V55 γ_1 , T79 γ_2 , and A85 β . The first two methyls are at the C-terminal end of the disordered linker connecting the WW and PPIase domains. The latter two are just after the flexible catalytic loop, at the N-terminal end of the long α helix –1. The increased mobility of these side chains may reflect loosening at a “flexure point” to facilitate rearrangements of the catalytic loop upon ligand binding. Such rearrangements are evident in X-ray crystal structures (9, 14), and implicated by our previous NMR exchange—broadening measurements (6).

Ligand binding caused consistent stiffening ($\Delta S^2_{\text{axis}} > 0$) at methyl axes of L61 δ_1 , I93 δ_1 , A124 β , L141 δ_2 , and I156 γ_2 . Methyl axes stiffening for at least three interactions included L60 δ_2 , L106 δ_2 , M130 ϵ , V150 γ_1 , and I159 δ_1 . Critically, these methyls (except for A124 β) are within or adjacent to the substrate binding pocket (L60, L61, M130, I156, and I159), or the interdomain interface (I93, L141, V150). Moreover, all of these methyls belong to residues of the proposed hydrophobic conduit, and thus, strengthen the notion that the conduit stiffening is endemic to the ligand binding process of Pin1.

Several dimethyl residues (ILV) gave anticorrelated changes in flexibility upon ligand binding. In particular, L122 δ_2 , V150 γ_2 , and I156 δ_1 consistently loosened upon ligand binding, whereas their stereo-related partners L122 δ_1 , V150 γ_1 , and I156 δ_2 consistently stiffened. These anticorrelated changes suggest an asymmetric reorganization of local steric interactions. The asymmetry could enhance contacts with one methyl, decreasing the mobility of its symmetry axis, while simultaneously enhancing the mobility of the other. L122 δ_1 and δ_2 are candidates for this scenario, because they make nonequivalent van der Waals contacts with the substrate proline ring (14).

Residues Showing Dynamic Changes Are Highly Conserved. If the above changes in side-chain flexibility are relevant for Pin1 substrate binding, then we expect conservation of the constituent residues among Pin1 homologs. We identified 43 highly conserved residues ($\geq 90\%$) from the sequence alignment of Pin1 homologs (*SI Text*). We compared these residues with those showing consistent losses or gains of flexibility. All residues that showed a consistent loss or gain of flexibility were highly conserved ($\geq 90\%$), with the exception of I93 and A124. Most of these

Fig. 5. Side chains showing consistent changes in flexibility upon ligand binding belong to highly conserved residues. (A) Blue spheres are methyl symmetry axes that consistently loosen ($\Delta S^2_{\text{axis}} < 0$), red spheres are methyl axes that consistently stiffen ($\Delta S^2_{\text{axis}} > 0$). (B) Green spheres are methyl-bearing groups conserved at $\geq 90\%$.

were the conduit residues L60, L61, M130, L141, V150, I156, and I159. Thus, there is a strong overlap between residues that are evolutionarily conserved, and those with side chains showing consistent binding-related changes in flexibility (Fig. 5).

Discussion

Comparing the dynamic consequences of binding *cis* versus *trans* conformers has been challenging for peptidyl-prolyl isomerases. We have answered this challenge for the human PPIase Pin1, by comparing how three related ligands—a substrate peptide FFpSPR and two inhibitor analogs locked in the *cis* and *trans* conformations—bind to Pin1 and alter its subnanosecond side-chain dynamics. The ligands differ at the core pS-P linkage. The substrate (FFpSPR) linkage undergoes *cis-trans* isomerization whereas the inhibitors cannot; otherwise, the composition and length of the three ligands are identical. We can therefore attribute the different dynamic responses to different conformational preferences of the pS-P linkage. The locked inhibitors are proxies of the substrate *cis* and *trans* ground states, and provide a unique opportunity to compare the effects of these two input conformers on Pin1 functional motions.

Chemical shifts showed different binding modes for the *cis* and *trans* inhibitors. The *cis* inhibitor is a nearly exclusive binder of the PPIase domain, whereas the *trans* inhibitor can bind both domains. The *cis* binds the PPIase active site more tightly; hence, saturating amounts of the *cis* inhibitor can knock off the *trans* inhibitor, which then binds just the WW domain. This suggests the design of fragment-linked inhibitors based on *cis* and *trans* binding cores targeting the PPIase and WW domains, respectively.

The methyl ^2D relaxation studies show that the substrate FFpSPR alters side-chain flexibility along a conduit of conserved hydrophobic residues linking the domain interface to the PPIase domain catalytic site. These alterations include both increases and decreases, with decreases (stiffening) dominating for the conduit residues that are less solvent accessible. This conduit overlaps with that observed in our previous study (6) of Pin1 interacting with a different substrate. The emergence of this conduit response for two substrates with different sequences and lengths (FFpSPR versus EQPLpTPVTDL) suggests that it is intrinsic to Pin1 substrate recognition.

The locked inhibitors also induced conduit stiffening; thus, the stiffening reflects substrate binding rather than catalysis. However, the stiffening is more pronounced upon *cis* inhibitor binding; hence, the conduit response is stereoselective. Also, backbone NH $J(0)$ and side-chain $^{13}\text{C}_{\text{methyl}}$ relaxation corroborate this selectivity. Such selectivity may help route different input conformations to different signaling pathways.

Our K_d analyses showed that the PPIase domain of full-length Pin1 binds the *cis* locked inhibitor more tightly than the *trans*.

Thus, tighter binding and conduit stiffening may be linked. Moreover, the truncated Pin1-PPI binds the *cis* inhibitor with slightly more affinity than the PPIase domain of full-length Pin1. This suggests that domain interactions can tune the breadth of active site conformations relevant for binding. Presumably, the PPIase active site in full-length Pin1 is optimized to bind the substrate transition state; this configuration may resemble *cis* more than *trans* (hence, the higher *cis* affinity), rather than being completely *cis* (hence, the higher affinity of Pin1-PPI versus full-length Pin1). This sparks interest in exploring transition-state analogues.

The Pin1 WW domain has been viewed as a docking module that enhances Pin1 specificity for pS/T-P motifs, and a modulator of PPIase activity due to the increase of local substrate concentration. Yet, mutations at the domain interface, in either the WW or PPIase domain, can reduce Pin1 activity as demonstrated by its inability to rescue yeast ESS1 knockouts (24). Together with the studies here, this suggests a parallel WW domain function: Its interaction with the PPIase domain can also tune the binding affinity of the remote (approximately 12 Å distance) PPIase domain catalytic site via an intraprotein signaling mechanism. Because the ^{15}N - ^1H spectra of *cis* saturated PPIase domain were nearly identical in the presence and absence of the WW domain, this putative signaling mechanism does not invoke dramatic conformational change. New insight comes from our dynamics studies: WW domain binding can modulate the changes in side-chain flexibility, in particular, those along the conduit bridging the domain interface and the PPIase active site. Thus, whereas the intraprotein signaling may involve subtle structural changes, it may also involve propagated changes in flexibility (25). Precedent for such propagation includes millisecond motions for interdomain allostery (26), and subnanosecond motions for single domain allostery (27). This work expands the range of responses by suggesting that propagated changes in subnanosecond flexibility can assist interdomain communication within modular systems.

Given the above, conduit stiffening may serve to alter the breadth of conformational fluctuations at one functional site, upon ligand binding at another. This would be consistent with the disparity of time scales: The conduit motions are on the subnanosecond time scale, whereas PPIase activity occurs on the μs -ms time scale. The rapid conduit response would act as a high-speed communication link between the two functional sites of Pin1, in anticipation of the less frequent catalytic events.

Methyl-bearing residues are often highly conserved in proteins, because they provide a stabilizing hydrophobic core. Among Pin1 homologs, many of the highly conserved methyls also show consistent changes in mobility across four Pin1-ligand interactions (Fig. 5). Hence, their conservation likely reflects dual purposes: (i) maintaining overall protein stability; and (ii) providing a network of steric interactions, such as the conduit, that can enable intraprotein signaling via changes in flexibility. In fact, (ii) may be a general fortuitous consequence of ligand-induced stabilization of a protein hydrophobic core with spatially separate functional sites. Further testing of the conduit hypothesis will require mutating conduit residues followed by binding and dynamics studies.

In conclusion, our studies extend the function of conformational mobility in Pin1, a modular protein recruited by numerous signaling proteins. The dynamic responses of Pin1 are stereoselective, and couple distinct functional sites. These dynamic responses entail flexibility changes that enable interactions between the modular domains to alter the interaction properties at the distal active site. Many proteins are modular and flexible, and thus, may exploit the dynamic gating strategies disclosed by Pin1.

Materials and Methods

Sample Preparation. Full-length Pin1 was overexpressed and purified as described previously (6); the truncated Pin1-PPI (residues 40–163) followed a similar protocol (SI Text). The final NMR buffer was 30 mM imidazole- d_4 —pH 6.6 with 30 mM NaCl, 0.03% NaN_3 , 5 mM DTT- D_{10} and 90% H_2O /

10% D₂O. Side-chain methyls and backbone nuclei were assigned at 295 K using standard triple-resonance experiments (6). The locked inhibitors and substrate FFpSPR were synthesized and purified as described previously (7, 8, 12). Further details regarding samples and ligand titrations are in [SI Text](#).

NMR Relaxation Experiments and Analyses. Deuterium relaxation rates $R_{1\rho}$ (²D) and R_1 (²D) for CDH₂ methyl isotopomers of full-length Pin1 and Pin1-PPI were measured with established pulse schemes (28, 29) at 16.4 and 18.8 T (700.13 and 800.13 MHz ¹H Larmor frequencies, respectively), 295 K. The

²D hard-pulses were applied at 1.78 kHz whereas the spin-locks were applied at reduced strengths of 1.2 kHz. Backbone amide R_1 (¹⁵N), R_{2-CPMG} (¹⁵N) and the steady-state ¹HN-¹⁵N NOE parameters were measured at 16.4 T using established pulse schemes (30, 31). Further details for ²D, ¹⁵N, and ¹³C relaxation and data analysis are in [SI Text](#).

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