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Conformational Selection in the Recognition of Phosphorylated Substrates by the Catalytic Domain of Human Pin1

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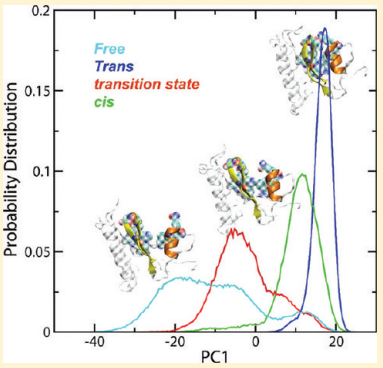
Supporting Information

ABSTRACT: Post-translational phosphorylation and the related conformational changes in signaling proteins are responsible for regulating a wide range of subcellular processes. Human Pin1 is central to many of these cell signaling pathways in normal and aberrant subcellular processes, catalyzing *cis*−*trans* isomerization of the peptide ω-bond in phosphorylated serine/threonine-proline motifs in many proteins. Pin1 has therefore been identified as a possible drug target in many diseases, including cancer and Alzheimer's. The effects of phosphorylation on Pin1 substrates, and the atomistic basis for Pin1 recognition and catalysis, are not well understood. Here, we determine the conformational consequences of phosphorylation on Pin1 substrate analogues and the mechanism of recognition by the catalytic domain of Pin1 using all-atom molecular dynamics simulations. We show that phosphorylation induces backbone conformational changes on the peptide substrate analogues. We also show that Pin1 recognizes specific conformations of its substrate by conformational selection. Furthermore, dynamical correlated motions in the free Pin1 enzyme are present in the enzyme of the enzyme−substrate complex when the substrate is in the transition state configuration, suggesting that these motions play significant roles during catalytic turnover. These results provide a detailed atomistic picture of the mechanism of Pin1 recognition that can be exploited for drug design purposes and further our understanding of the synergistic complexities of post-translational phosphorylation and *cis*−*trans* isomerization.



Conformational transitions and the switching of biomolecules play important roles in subcellular processes. These processes generally involve recognition and transient interactions between biomolecules. The local switching of the isomeric state of the peptide bond preceding proline (also known as the ω-bond angle) from *trans* to *cis*, or vice versa, is known to bring about conformational changes in proteins and regulate a wide range of subcellular processes.[1] When the prolyl ω-bond is in the *trans* configuration, the protein may have a particular function that is altered when the prolyl ω-bond is switched to the *cis* configuration, thereby allowing biological signaling processes to utilize the additional conformational diversity created by the two possible isomeric states of the ω-bond angle. However, this signaling mechanism is very slow on the biological time scale and is therefore regulated by ubiquitous peptidyl prolyl *cis*−*trans* isomerases (PPIases), accelerating *cis*−*trans* isomerization of the prolyl peptide bond by several orders of magnitude.[2] The role of *cis*−*trans* isomerization in the interleukin tyrosine kinase (Itk) SH2 domain,[3] ligand gated 5-HT3 ion channel,[4,5] HIV capsid formation,[6] and protein folding[7] are a few familiar examples. Unlike the two well-known PPIases, cyclophilins[8] and FKBPs,[9] the more recently discovered human Pin1 is a phosphorylation-dependent PPIase.[10] Pin1 is specific for prolyl ω-bonds when the preceding residue is a phosphorylated serine (pSer) or phosphorylated threonine (pThr), combining the regulatory role of post-translational phosphorylation and conformational switching of prolyl ω-bonds (Figure 1). In particular, Pin1 is involved in mitosis, various cancers, and Alzheimer's disease.[11,12] Additionally, many important proteins in humans have been identified as substrates for Pin1,[13] including RNA polymerase II.[14]

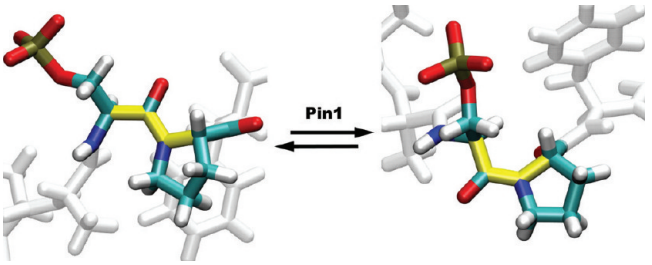


Figure 1. Pin1 catalyzes *cis*−*trans* isomerization of the peptide backbone ω-bond angle of proline when the preceding residue is a phosphorylated serine or phosphorylated threonine. The ω-bond angle is colored yellow when it is in the *trans* configuration (left) and in the *cis* configuration (right).

The discovery of Pin1 has provided additional evidence of the involvement of PPIases in cell signaling.[15] Pin1 plays an important role in the G_2−M phase transition in mitosis through its interaction with topoisomerase.[11] This interaction allows

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Pin1 to localize chromatin in the G_2−M phase of mitosis. Pin1 also interacts with many other important mitotic proteins such as CDC25C, EM11, and WEE1.[15] Inhibition or deletion of Pin1 leads to mitotic catastrophe and chromatin condensation; therefore, Pin1 is a key enzyme in mitosis. During the G_1−S phase transition, Pin1 interacts with Cyclin D1, creating stabilization and nuclear localization for the transition, and the level of Pin1 in normal cells is elevated because many of its protein substrates control this transition. In various cancers (breast, thyroid, and prostate), Pin1 and its substrate, Cyclin D1, are overexpressed, indicating that both play an important role in oncogenesis.[13] Consequently, Pin1 has been an effective early diagnostic marker in prostate cancer.[16] Pin1 is viewed as a "double-edged sword".[12] In normal cells, phosphorylation of Ser/Thr-Pro motifs occurs in many signaling pathways. Similarly, in cancer cells, phosphorylation of Ser/Thr-Pro motifs follows oncogenic signaling. As a result, Pin1 regulates proteins that are involved in both promotion and suppression of oncogenesis and has been identified as a target for drug design.[16] In Alzheimer's disease, it is believed that Pin1 actually protects against the disease, because it is deactivated in the early stages of the illness through oxidative modification.[11] More specifically, phosphorylation and dephosphorylation of tau protein regulate the assembly of microtubules in neuronal cells, and Pin1 regulates *cis*−*trans* isomerization of numerous pSer/pThr-Pro motifs in tau protein.[17] When Pin1 is depleted, tangling of hyperphosphorylated tau takes place, which is one of the hallmarks of Alzheimer's disease, eventually resulting in cell death.[18] Clearly, deciphering the exact role of Pin1 and its mechanism of action at the atomic level will provide a deeper understanding of many diseases and will help in the design of new classes of drug candidates.

The distinguishing characteristic of Pin1 compared to the other PPIases is that it is selective for the peptide ω-bond of pSer/pThr-Pro motifs. Therefore, phosphorylation of Ser/Thr-Pro motifs facilitates the interaction between Pin1 and its protein substrates. It is known that phosphorylation perturbs the free energy landscape of proteins.[19−21] However, the effects of phosphorylation on the conformational landscape of Pin1 substrates and how phosphorylation facilitates recognition by Pin1 are not well understood. In addition, X-ray crystal structures of Pin1 bound to its natural substrates have not been determined. Therefore, a detailed atomistic understanding of how Pin1 interacts with its substrate is lacking. Also, knowledge of the interactions of Pin1 with its natural substrates could improve our understanding of the mechanism of *cis*−*trans* isomerization of Pin1 and possibly other isomerases, a mechanism that is still not well understood and controversial.[2,22] In understanding the effects of phosphorylation on the conformation of Pin1 substrates and the role phosphorylation plays in the recognition by Pin1, we focus here on studying the effects of phosphorylation on the conformational preferences of well-studied substrate analogues, Ace-Ala-Ala-X-Pro-Phe-Nme, where X is pSer and pThr, and their interactions with Pin1 using all-atom molecular dynamics (MD) simulations in explicit water. Simulations of the free substrate analogues and one of the enzyme−substrate complexes were conducted using accelerated molecular dynamics (aMD),[23] which allows for a very efficient and thorough sampling of the configurational space and the slow *cis*−*trans* isomerization of the prolyl ω-bond.

METHODS

All simulations were conducted using the Amber 10 suite of programs[24] and the modified parm99SB[25] version of the force field of Cornell el al.,[26] with the reoptimized dihedral parameters for the peptide backbone ω-bond.[27] The NPT ensemble at a pressure of 1 bar and a temperature of 300 K was used for all the simulations with the explicit TIP3P[28] water model, and the SHAKE[29] algorithm was used to constrain all bonds involving hydrogen. The Langevin thermostat was used for regulating the temperature of the system to 300 K with a collision frequency of 1.0 ps^{-1}. Short-range nonbonded interactions were calculated with a cutoff of 9 Å, and all long-range interactions were calculated using the particle mesh Ewald summation.[30] A time step of 2 fs was used to integrate Newton's equations of motion.

Accelerated Molecular Dynamics of Pin1 Substrate Analogues. The substrate analogues, Ace-Ala-Ala-X-Pro-Phe-Nme, were built with XLEAP, where X was Ser, Thr, pSer, or pThr. The parameters used for the pSer and pThr residues were developed by Homeyer et al.[31] The dianionic form of pSer and pThr was used in this study because it was shown that at around neutral pH the phosphate group is deprotonated and Pin1 prefers to bind its substrates in the dianionic form even at low pH.[32,33] The substrates were placed in a cubic periodic water box with the edges of the box at least 10 Å from any part of the substrates. Two Na^+ ions were added to the systems of phosphorylated substrates to attain electrostatic neutrality. The systems were then equilibrated with a series of minimization and molecular dynamics simulations. Each equilibrated system was then simulated for 260 ns using accelerated molecular dynamics. A boost energy, E, of 127 kcal/mol and an α of 20 kcal/mol were used for the acceleration. Accelerated molecular dynamics was implemented in a modified version of pmemd in the AMBER 10 suite of programs. Each simulation was then repeated 10 times, each with a different random seed. A snapshot of the simulation was written to the trajectory file every five steps. A high frequency of data collection was used to reduce errors due to reweighting.[34] Ptraj and Matlab were used to analyze the data.

Molecular Dynamics Simulations of Pin1 and Pin1−Substrate Complexes. The starting structure for this study was a 1.7 Å resolution X-ray crystal structure of an inhibitor-bound complex of Pin1 with Protein Data Bank (PDB) entry 2Q5A.[35] Only the catalytic domain was used. The WW domain was removed, because it does not affect the catalytic activation and binding of the catalytic domain.[33] The inhibitor was removed to prepare the system for the free Pin1 enzyme. To prepare the enzyme−substrate complexes, the peptidomimic inhibitor was modified to the sequence of the substrate analogues. The peptidomimic inhibitor already provided the peptide backbone template, and the side chains of the non-natural amino acids were modified to amino acids in our sequence by retaining the common atoms. Xleap was used to add the missing atoms. The systems were solvated in a periodic truncated octahedron water box with the edges of the box at least 10 Å from any part of the solute. The systems were then neutralized with counterions. The systems were equilibrated with a series of minimization and molecular dynamics simulations, initially applying a harmonic constraint only on the solute with a force constant of 200 kcal mol^{-1} $Å^{-2}$ and reducing it by half for each subsequent equilibration step until it equals 25 kcal mol^{-1} $Å^{-2}$. A final molecular dynamics

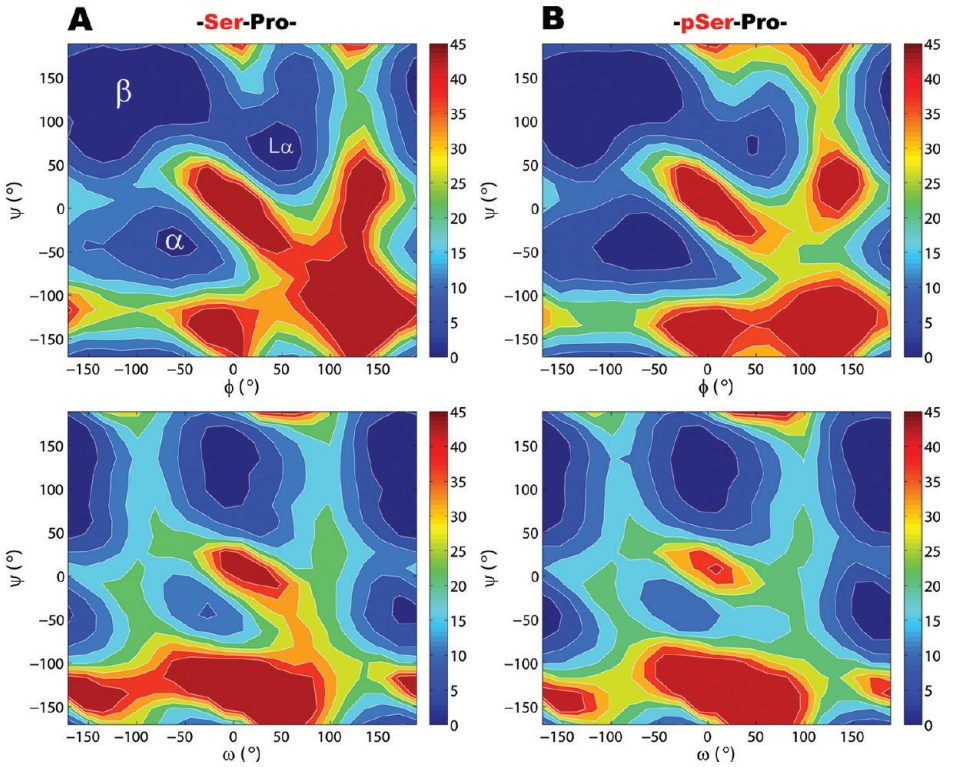

Figure 2. Contour plot in kilocalories per mole of the conformational space of unphosphorylated (Ser) and phosphorylated serine (pSer) in the free substrate analogue, Ace-Ala-Ala-pSer-Pro-Phe-Nme, of Pin1. (A) $\varphi-\psi$ or Ramachandran plot (top) of Ser, with the relevant regions labeled as α (α-helical region), β (β-region, broadly defined), and Lα (left-handed helical region), and $\omega-\psi$ plot (bottom) of Ser and the preceding ω-bond angle of the Ser-Pro motif. (B) $\varphi-\psi$ or Ramachandran plot (top) of pSer and $\omega-\psi$ plot (bottom) of pSer and the preceding ω-bond angle of the pSer-Pro motif.

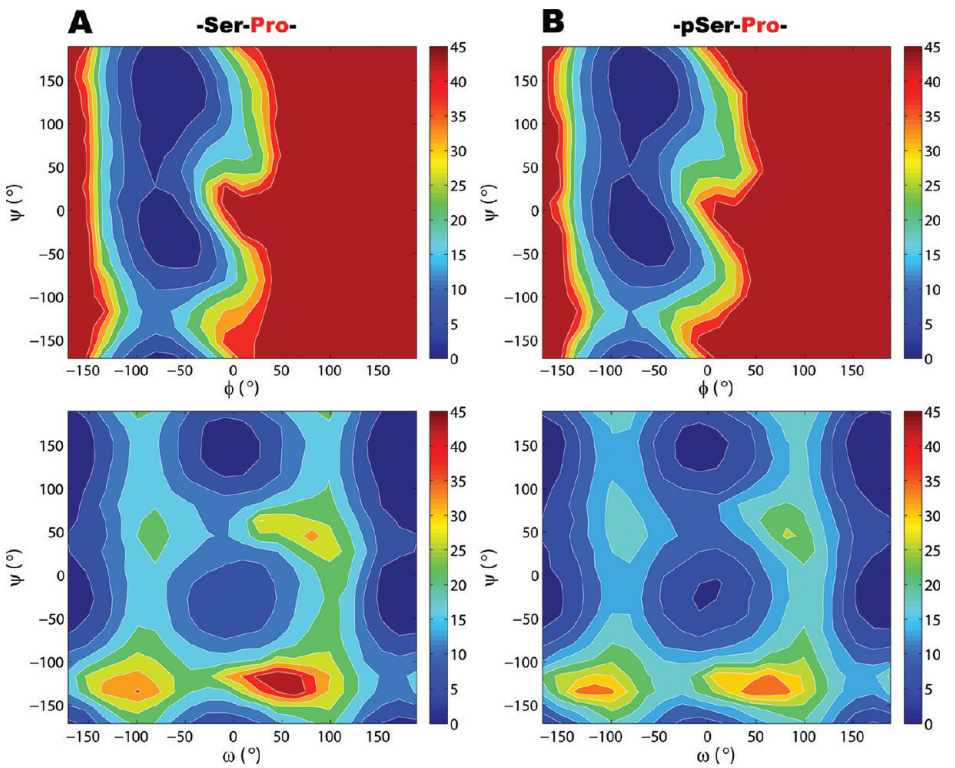

Figure 3. Contour plot in kilocalories per mole of the conformational space of Pro in the free phosphorylated and unphosphorylated substrate analogue, Ace-Ala-Ala-pSer-Pro-Phe-Nme, of Pin1. (A) $\varphi-\psi$ or Ramachandran plot (top) of Pro of the unphosphorylated substrate and $\omega-\psi$ plot (bottom) of proline and the preceding ω-bond angle of the unphosphorylated Ser-Pro motif. (B) $\varphi-\psi$ or Ramachandran plot (top) of Pro of the phosphorylated substrate and $\omega-\psi$ plot (bottom) of proline and the preceding ω-bond angle of the pSer-Pro motif.

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simulation was conducted without any harmonic constraints. The systems were then simulated for 110 ns each. The first 10 ns was discarded as additional equilibration. The resulting conformation of the peptide ω-bond of the X-Pro motif of the substrates was in the *cis* configuration. The transition state and *trans* configurations of the Pin1−substrate complexes were generated by slowly converting the ω-bond angle from *cis* (0°) to transition state (90°) and then to *trans* (180°) using the angle restraint in AMBER with a force constant of 1000 kcal mol^{-1} rad^{-2}. During the simulation of the Pin1−substrate complex when the ω-bond angle of the substrate is in the transition state configuration, the ω-bond angle was held using the angle restraint between 85° and 95° with a force constant of 1000 kcal mol^{-1} rad^{-2}. The principal component analysis[36] was conducted by combining the trajectories of free Pin1 and Pin1 in the different states of the substrates (*cis*, *trans*, and transition state) using the Ptraj module in the AMBER suite of programs. The accelerated molecular dynamics simulation of the Pin1−substrate (X = pSer) complex was conducted for 260 ns starting from the fully equilibrated structure by applying the acceleration to the substrate in the active site of Pin1. A boost energy, *E*, of 127 kcal/mol and an α of 15 kcal/mol were used for the acceleration. A snapshot of the simulation was written to the trajectory file every five steps.

RESULTS AND DISCUSSION

We have conducted extensive sampling of the conformational space of Pin1 substrate analogues, Pin1−substrate complexes, and free Pin1, using all-atom molecular dynamics simulations in explicit water. These simulations have allowed us to study the effects of phosphorylation on the conformations of the substrates, containing the pSer/pThr-Pro motifs, and the mechanism of recognition by the catalytic domain of Pin1. Post-translational modification of proteins by phosphorylation is intricately involved in many normal and aberrant signaling pathways in biology. Therefore, understanding the exact role of phosphorylation in biology is a key to treating many diseases.

Effects of Phosphorylation on the Conformational Equilibrium of Pin1 Substrate Analogues. Accelerated molecular dynamics simulations were conducted on Pin1 substrate analogues (Ace-Ala-Ala-X-Pro-Phe-Nme), where X was Ser, Thr, pSer, or pThr. Each substrate was simulated for 260 ns, and each simulation was repeated 10 times for a total of 2.6 μs. The results of the conformational sampling of the Ser/pSer-Pro motifs of the free substrates are summarized in Figures 2 and 3. The results for Thr/pThr-Pro are similar to those for Ser/pSer-Pro and are summarized in Figures S1 and S2 of the Supporting Information. The Ramachandran or $\varphi-\psi$ plots for X = Ser and Thr reveal that the right-handed α-helical region ($\varphi \sim -75°$, and $\psi \sim -50°$) for serine is slightly more populated than that for threonine. The left-handed helical regions for both X = Ser and Thr are reasonably populated, although the left-handed basin for X = Ser is slightly broader. Nonetheless, the β-region for both X = Ser and X = Thr is the most populated, with a very broad basin. These results are similar to those obtained by Lee et al.[37] in their study of the Gly-Ser-Ser-Ser peptide, but with some differences. They concluded that prior to phosphorylation the backbone of serine is mainly in the β-region and polyproline II region, with very little or no α-helical conformation. In this study, a significant population of the α-helical conformation is seen prior to phosphorylation, although the β-conformation dominates.

An earlier NMR study by Tholey et al.[38] showed that phosphorylation of the Gly-Ser-Ser-Ser peptide results in changes to the ^3J coupling constants of the backbone angles, indicating a change in the equilibrium conformation of the peptide. These results were later supported by Shen et al.[39] who used atomistic Brownian dynamics simulations. They showed that phosphorylation increases the α-helical population of the peptide backbone conformation. Similar results were also obtained for the Thr-pSer-Pro-Ile peptide as well as for the pSer/pThr-Pro dipeptide.[40,41] A similar effect is also observed here for Pin1 substrate analogues. Upon phosphorylation, i.e., when X = pSer or pThr, there are two main observations: a considerable increase in the population of the right-handed α-helical conformation and a decrease in the population of the left-handed helix for both the pSer- and pThr-containing substrates as compared to the unphosphorylated substrates. The population of the β-region in both substrates, however, does not change significantly upon phosphorylation (Figure 2B and Figure S1B of the Supporting Information). In this study, the majority of the increase in the population of the α-helical region has been at the expense of the left-handed region. The population of the left-handed helical region when X = pThr is almost wiped out upon phosphorylation, while a small population of the left-handed helical conformation is maintained when X = pSer. Also, phosphorylation decreases the level of the left-handed helical region of the flanking residues of X-Pro but does not significantly alter their $\alpha-\beta$ equilibria, as summarized in Figures S3 and S4 of the Supporting Information. Therefore, the results suggest that the effects of phosphorylation on the backbone conformation of the substrate are mainly local but can propagate beyond the phosphorylation site. One major difference between the two types of substrates is that the high-energy or "forbidden" region (energies of more than 40 kcal/mol) is larger when X = Thr and pThr than when X = Ser and pSer. Therefore, the Thr/pThr-Pro motif seems to be less flexible than the Ser/pSer-Pro motif. Beck et al.[42] have shown that threonine-containing motifs covers less space on the Ramachandran plot than serine-containing motifs. Our results show that this is also true for the phosphorylated motifs, and these subtle differences between the conformations of pThr-Pro and pSer-Pro motifs and their effects on the substrate analogues of Pin1 could have implications for the slight differences in their recognition and catalysis by Pin1. For example, it appears that pThr-Pro-containing substrates binds better to Pin1 than pSer-Pro-containing substrates,[43] but the catalytic activity of Pin1 for pSer-Pro-containing substrate seems higher than that of pThr-Pro substrates.[44]

Previously, it was shown that there is a dependency of the ω-bond angle on the backbone ψ-angle of Pro for X-Pro motifs.[40,45] It turns out that there is also a dependency of the ω-bond angle on the backbone ψ-angle of X. In Figures 2 and 3 (bottom), we have plotted the ψ-angle of Ser and pSer as a function of the ω-bond angle and that of Pro for the unphosphorylated and phosphorylated substrate analogues, respectively. As expected, the predominant isomer for the ω-bond angle is *trans* for all the substrates, as can be seen from the $\omega-\psi$ spaces in Figures 2 and 3. Interestingly, the α-helical and β-regions defined by the ψ-angle of X and Pro can easily interconvert and are almost equally populated when the ω-bond angle is in the *trans* configuration for the unphosphorylated substrates. However, the β-region of the ψ-angle of X and Pro is more populated when the ω-bond angle is in the *cis*

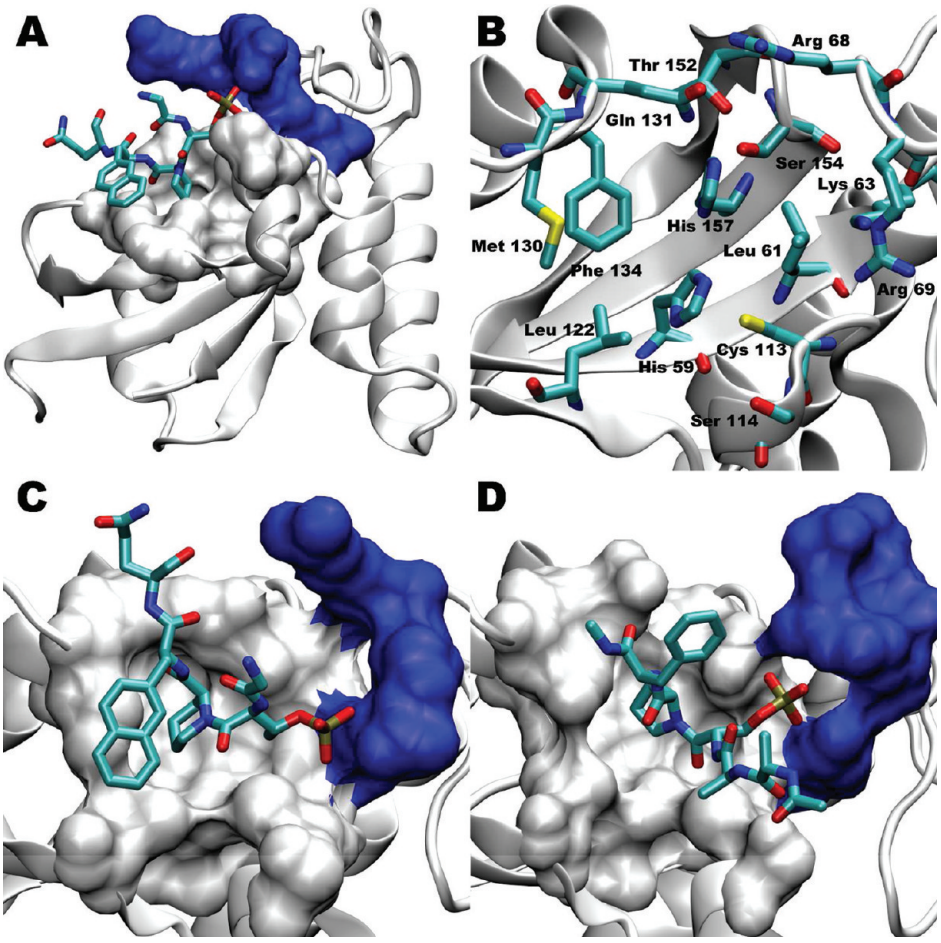

Figure 4. Structure and binding site of the catalytic domain of Pin1. (A) X-ray crystal structure (PDB entry 2Q5A) of the catalytic domain of Pin1 (white), with a peptidomimetic in the active site. The active residues are shown using surface representation. (B) Residues that make up the active sites. (C) Peptidomimetic inhibitor interacting with the proline binding pocket and the phosphate binding residues (Lys 63, Arg 68, and Arg 69) (blue). (D) Substrate analogue, Ace-Ala-Ala-pSer-Pro-Phe-Nme, in the active site interacting with the proline binding pocket and the phosphate binding residues (Lys 63, Arg 68, and Arg 69) (blue).

configuration for the unphosphorylated substrates than the α-helical region. Also, the barrier separating the α-helical and β-regions is smaller when the ω-bond angle is in the *trans* configuration than when it is in the *cis* configuration; therefore, it is easier for X-Pro motifs to change backbone conformations when they are in the *trans* configuration. The barrier separating the α-helical and β-regions, when the ω-bond angle is in the *trans* configuration, is slightly lower for Pro than for X.

Upon phosphorylation, there is a slight reduction in the barrier separating the α-helical and β-regions for Pro when the ω-bond angle is in the *cis* configuration, creating a thermodynamically stable valley for the conformational transition. The same barrier for X does not change much upon phosphorylation. Therefore, the results suggest that phosphorylation also can act to facilitate the conformational transition between the α-helical and β-regions for Pro when the ω-bond angle is in the *cis* state. In addition to lowering the barrier between the α-helical and β-regions of Pro when the ω-bond is in the *cis* configuration, phosphorylation also increases the α-helical population of Pro, as shown in Figure 3.

Conformational Selection in Pin1 Recognition. The overall *cis*–*trans* equilibrium and barrier along the ω-bond are only slightly affected by phosphorylation. The barrier from the *trans* configuration to the *cis* configuration is calculated to be ~20 kcal/mol for both the unphosphorylated and phosphorylated substrate analogues, very similar to experimentally measured values.[32] Because phosphorylation does not seem to greatly affect the *cis*–*trans* barrier, it can be argued that phosphorylation renders the Ser/Thr-Pro motifs almost unrecognizable by the other ubiquitous and promiscuous isomerases (cyclophilins and FKBPs), and specific for Pin1 recognition. What role does phosphorylation therefore play in Pin1 recognition and specificity?

X-ray crystal structures of natural substrates or substrate analogues bound to Pin1 have not been determined. However, structure-based drug design has provided several lead compounds for targeting Pin1,[46] and X-ray crystal structures for some of those compounds in the active site of Pin1 have been determined.[35,47,48] To study the mechanism of Pin1 recognition of its natural substrates, we have modeled the enzyme–substrate complex from a 1.7 Å resolution X-ray crystal structure of a phosphorylated peptidomimetic inhibitor bound in the active site of Pin1,[35] with PDB entry 2Q5A, as shown in Figure 4A. The active site residues of Pin1 are shown in Figure 4B. The peptidomimetic inhibitor serves as an ideal starting point for modeling the natural substrate because it readily provides the template for the peptide backbone, and non-natural amino acid side chains can be easily modified to natural amino acids by keeping the common atoms. We modified the Pin1-bound inhibitor to the substrate analogues

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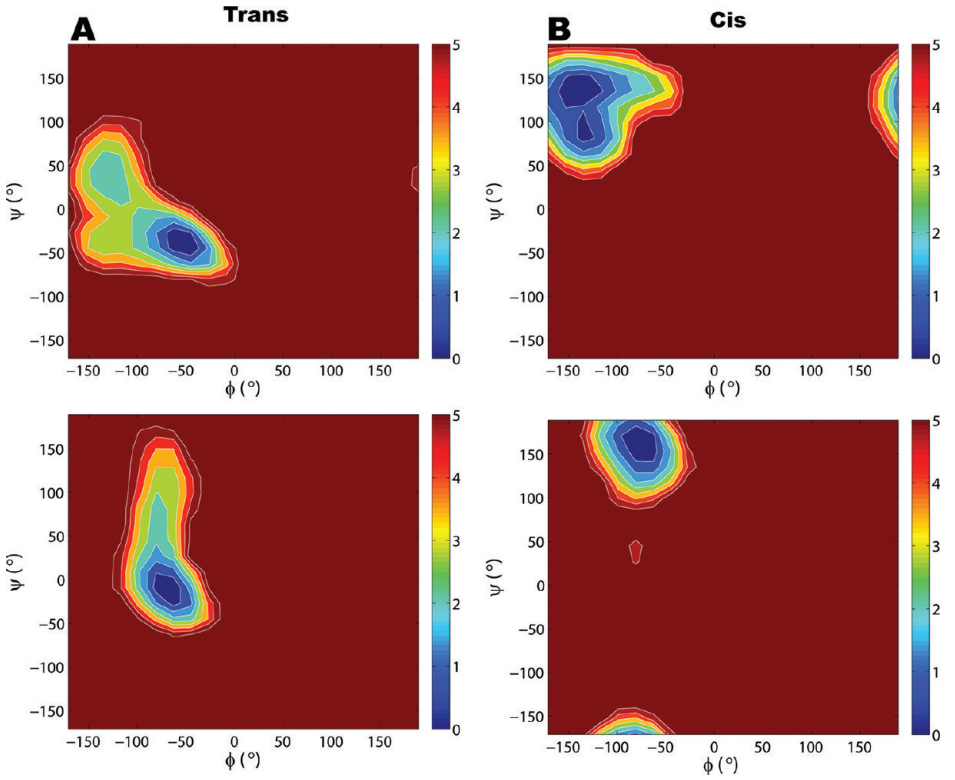

Figure 5. Contour plot in kilocalories per mole of the $\varphi-\psi$ space or Ramachandran plot of the pSer-Pro motif of the substrate analogue, Ace-Ala-Ala-pSer-Pro-Phe-Nme, in the active site of the catalytic domain of Pin1 for pSer (top) and Pro (bottom) (A) when the ω-bond angle of the pSer-Pro motif is in the *trans* configuration and (B) when the ω-bond angle of the pSer-Pro motif is in the *cis* configuration.

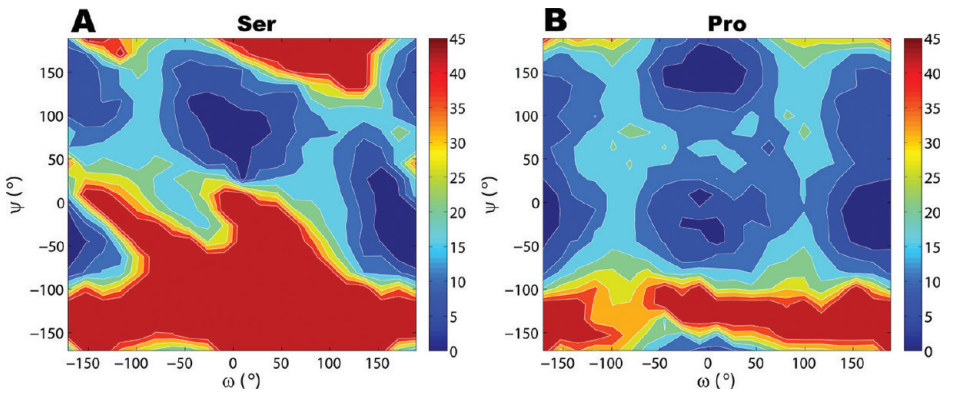

Figure 6. Contour plot in kilocalories per mole of the $\omega-\psi$ space of (A) pSer and (B) Pro of the pSer-Pro motif of the substrate analogue, Ace-Ala-Ala-pSer-Pro-Phe-Nme, when bound in the active site of the catalytic domain of Pin1.

with X = pSer or X = pThr. The complexes were then solvated with explicit water, minimized, and equilibrated for 10 ns. The final equilibrated structure of the complex between Pin1 and the substrate analogue when X = pSer is shown in Figure 4D. The active site can be broadly separated into two compartments. His 59, Leu 61, Cys 113, Leu 122, Met 130, Gln 131, Phe 134, Thr 152, Ser 154, and His 157 (residues on the left of Figure 4B) form the X-Pro binding pocket that mainly recognizes the side chain of proline. Lys 63, Arg 68, and Arg 69 form the binding pocket for the phosphate moiety of pSer that could form a clawlike structure around the phosphate moiety.[49] The configuration of the resulting X-Pro ω-bond angle is in the *cis* isomer ($\omega \sim 0°$) after the modification. Simulations of both complexes with the ω-bond angle of the X-Pro motif of the substrate analogues in the *cis* conformation were each extended for 100 ns. The ω-bond angle of the X-Pro motif of the substrates for both complexes was gradually converted to the *trans* (180°) conformation over a 1 ns MD simulation using angle restraints, and the complexes were allowed to relax with the ω-bond angle of the substrate in the *trans* conformation for an additional 10 ns. The *trans* complexes were also then simulated for an additional 100 ns.

Analyses of the backbone dihedral angles of the substrate when bound to Pin1 suggest that, in addition to recognizing the phosphate group of the substrate, Pin1 prefers specific backbone conformations of the substrate depending on the configuration of the ω-bond angle. When the ω-bond angle of the substrate is in the *cis* configuration, the backbone conformations of the X-Pro motifs are in the β-region, and this is true for both X = pSer and X = pThr as shown in Figure

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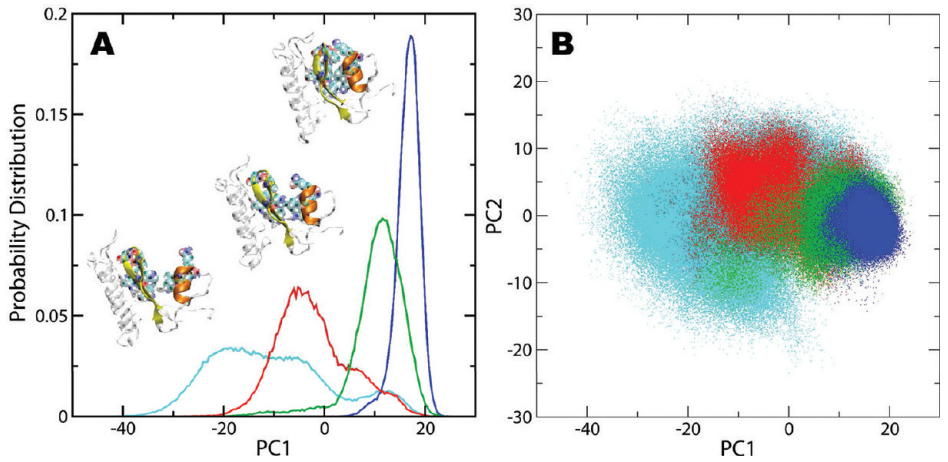

Figure 7. (A) Probability distribution of the first principal component (PC1) of the Pin1−substrate complex when the ω-bond angle of the pSer-Pro motif of the substrate is in the *trans* (blue), *cis* (green), and transition state (red) configurations and free Pin1 (cyan). The different conformations of free Pin1 along PC1 are shown as insets. The motion along PC1 is due to a hingelike motion between the helix (orange) and β-turn (yellow), with the proline binding pocket in the middle, colored by atom type (cyan for carbon, blue for nitrogen, and red for oxygen). (B) Plot of the first two principal components (PC1 and PC2) for the different states of the Pin1−substrate complex and free Pin1.

5 and Figure S5 of the Supporting Information. However, when the ω-bond angle of the substrates is in the *trans* configuration, the backbone conformations of the X-Pro motif of the substrates in the complexes are mainly in the α-helical region (Figure 5 and Figure S5 of the Supporting Information). The backbone conformation of the substrates is localized in the complex and is similar for both pSer- and pThr-containing substrates. The results therefore suggest that Pin1 recognizes its two different substrates in a similar fashion and selects a very small number of substates of the many different conformations that can be accessed by the free substrates. It is interesting to note that the backbone conformation of Pro, when the ω-bond angle is in the *cis* configuration for both free substrates (when X = pSer and pThr), is also mainly in the β region, and Pin1 recognizes this dominant conformation in the *cis* configuration. We also conducted accelerated molecular dynamics of the Pin1−substrate complex, when X = pSer, for 260 ns, to study *cis*−*trans* isomerization of the X-Pro ω-bond in the active site of Pin1. As seen in the results obtained from the normal molecular dynamics simulations, Pin1 prefers the substrate in the α-helical conformation when the ω-bond angle is in the *trans* configuration and the β-region when the ω-bond angle is in the *cis* configuration, as shown in Figure 6. The results further suggest that the ease of *cis*−*trans* isomerization along the ω-bond angle depends on the backbone conformation of Pro. The *cis*−*trans* barrier height on the positive side of the ω-bond angle when the backbone conformation of Pro is α-helical is lower than those of the other possible paths, also shown in Figure 6. These results therefore present compelling evidence that Pin1 recognition and binding, and possibly catalysis, rely not only on the electrostatic effects of the phosphate moiety but also on the ensuing changes in the secondary structure of the substrates. This interplay between the backbone conformations and isomeric states of the Pin1 substrates and Pin1 recognition could be very important for proper function. For example, proline-directed serine/threonine kinases and phosphatases phosphorylate and dephosphorylate Ser/Thr-Pro motifs, respectively, only if the ω-bond angle is in the *trans* configuration.[17,50,51] Therefore, Pin1 is critical to maintaining the *cis*−*trans* equilibria and regulating the function of its substrates and other proteins.

Interestingly, in addition to the recognition of specific conformations of the substrate analogues in the different states, the mechanism of Pin1 recognition is also based on conformational selection, which is believed to be a widespread mechanism in protein recognition.[52,53] We conducted principal component (PCA) analysis[36] on the simulations of free Pin1 and the substrate-bound complexes of Pin1, when the ω-bond angle of the substrate is in *trans*, *cis*, and transition state configurations. The simulations of the transition state of the substrate analogues bound to Pin1 were also conducted for 100 ns, using normal molecular dynamics, while the ω-bond angle was kept between 85° and 95° with an angle force constant restraint of 1000 kcal mol^{-1} rad^{-2}. Similar results were obtained for X = pThr; therefore, only the results for X = pSer are shown. Figure 7 shows the distribution of the first two eigenvectors of the slowest principal components (PC) projected back on the trajectories. The eigenvalues of the top five eigenvectors are 197.2, 23.2, 12.8, 11.5, and 8.9 Å^2; therefore, it can be seen that the first two PCs characterize almost 90% of the motions described by the top five PCs, and the first PC represents more than 70% of all the motions. Figure 7A shows the distribution of PC1 of Pin1 in the different states. Free Pin1 spans a large region of PC1 with approximately three peaks, representing the different conformations of Pin1 along PC1. However, it is interesting to see that the conformations of Pin1 that bind the substrate analogues when they are in the *cis*, *trans*, and transition state configurations are subsets of the free enzyme. The conformations of Pin1 that bind the *cis* and *trans* configurations of its substrate are similar, representing the least populated peak of free Pin1. The ensemble of conformations that binds the *trans* configuration has a distribution narrower than that of the *cis* configuration. However, the transition state configuration is recognized by a broader range of conformations that partly include that of the *cis* and *trans* configurations and, to a larger extent, one of the two dominant ensembles of conformations of the free enzyme. Therefore, the distribution of the conformations along PC1 that bind the transition state is much broader than that of the *cis* and *trans* configurations, as shown in Figure 7.

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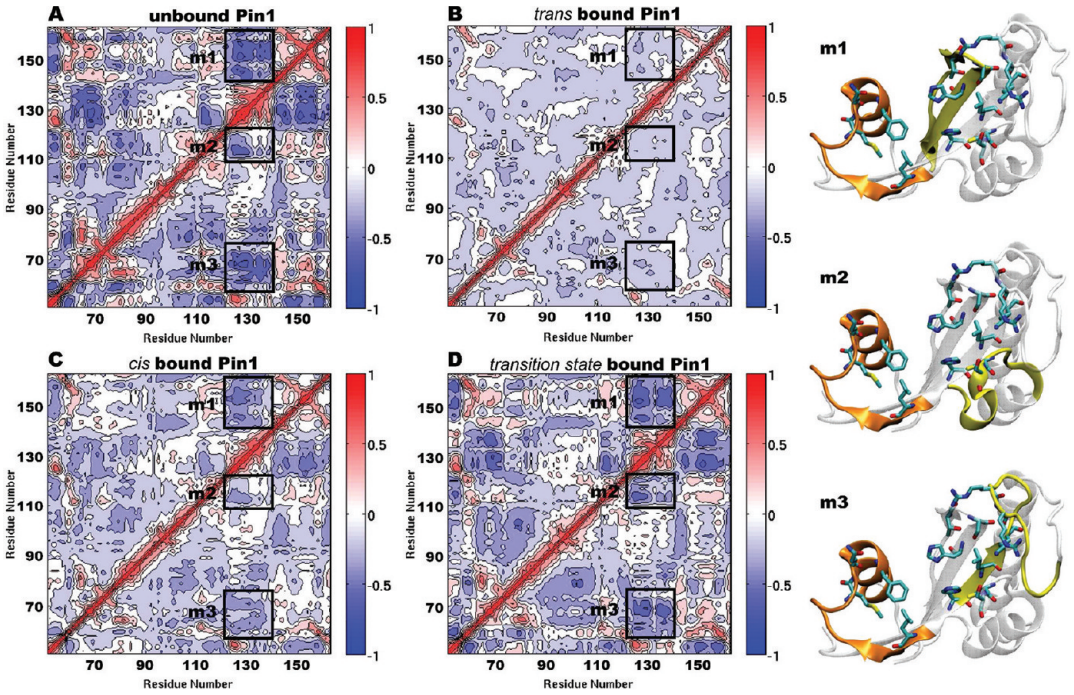

Figure 8. Dynamical cross-correlated motions of Pin1 residues in (A) free Pin1 and Pin1 bound to the substrate in the (B) *trans*, (C) *cis*, and (D) transition state configurations. The main correlated motions of Pin1 are labeled m1, m2, and m3, and the residues and domains involved in these motions are depicted at the right.

The motion of Pin1 that is most dominant and captured by the first principal component is also shown as insets in Figure 7. This motion represents a hingelike motion between an α-helix and a β-turn, with the proline binding pocket sitting between the two. Therefore, this hingelike motion modulates the active site and could possibly have a meaningful role to play in the catalytic mechanism of the enzyme. The different ensembles of conformations that recognize the different configurations of the substrate analogue interact slightly differently with the pSer-Pro motif. The side chain of Pro of the substrate forms a hydrophobic interaction with the proline binding pocket of Pin1 (Figure 4) when the substrate is in the *cis*, *trans*, and transition state configurations. Also, the phosphate moiety of the side chain of pSer always forms electrostatic interactions with the phosphate binding pocket (also Figure 4) irrespective of the state of the substrate. Arg 69 and Lys 63 form more stable interactions with the phosphate moiety than Arg 68, as shown in Figure S6 of the Supporting Information. Arg 69 and Lys 63 are more localized around the phosphate group, while Arg 68 is more mobile and rarely interacts with the phosphate group. Unlike the *cis* and *trans* states, the results suggest that the transition state of the substrate interacts a little more favorably with Arg 68. These results agree well with earlier mutational studies showing that Arg 69 is more important for binding than Arg 68.[33]

Dynamical and Correlated Motions in Pin1. The motion of Pin1 along the first principal component is shown to be the most dominant (Figure 7) and spans a large range in the free enzyme. This motion is dominant in the enzyme of the transition state-bound complex and also spans a relatively large range, as compared to that of the *trans*- and *cis*-bound complexes. This led us to speculate that in addition to the ability of the enzyme to stabilize the transition state during catalysis this dominant motion in the free enzyme could play a key role during catalytic turnover. This is interesting because the well-studied cyclophilin A, which is a *cis*−*trans* isomerase of a different family, has been shown to possess motions in the free enzyme that are present in the enzyme−substrate complex during catalytic turnover. These motions are believed to assist in catalysis by moving the substrate from one state to the other.[54−56] This dynamical phenomenon has also been observed in other enzymes,[57,58] including Pin1.[59−61] However, the effect of these dynamical motions on the catalytic rate and whether they provide any enhancement to the rate of catalysis as compared to the isomerization of the substrate (free in solution) are not clear and are still being debated.[62,63] What is clear is that these dynamical motions are generally coupled with the catalytic process, as the enzymes are very dynamic and have to alter their conformations to recognize the substrate in the different states, as observed in this work.

In addition to the dynamical motions represented by the first principal component, we have also calculated the dynamical correlated motions in free Pin1 and the substrate-bound complexes in the three different states, as shown in the dynamical cross correlation map in Figure 8. It is interesting and surprising to see that correlated motions in the free enzyme (labeled as m1, m2, and m3 in Figure 8) are also present in the enzyme conformations that bind the transition state. However, they are not present in the *trans*−enzyme complex and are only slightly present in the *cis*−enzyme complex. The correlated motions in free Pin1 are more similar to that of the transition state complex than those of the *cis* and *trans* complexes (Figure 8). All of the highlighted motions involve amino acids between positions 122 and 140 (colored orange in Figure 8) and three other parts of the enzyme (colored yellow in Figure 8). All of the correlated motions in one way or another involve residues that make up the active site of Pin1. The segment of residues 122−140 contains most of the residues that make up the proline binding pocket, including Phe 134 that is at the base of the cavity. The m1 correlated motion is between the majority of

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the proline binding site residues (Leu 122, Met 130, Glu 131, and Phe 134) and three other active site residues, Thr 152, Ser 154, and His 157, one of which (Ser 154) is actively involved in hydrogen bonding with the substrate when the substrate is in the *trans* and transition state configurations. The correlated motion depicted by m2 is again between the majority of the proline binding site residues (Leu 122, Met 130, Glu 131, and Phe 134) and two other active site residues, Cys 113 and Ser 114. Cys 113 can hydrogen bond with the substrate when it is in the *cis* configuration and also when it is in the transition state. The third correlated motion, m3, involves the proline binding pocket and the residues that make up the phosphate binding pocket (Lys 63, Arg 68, and Arg 69). Several of these residues have previously been identified as being important for catalysis using NMR relaxation experiments.[59−61] Our results therefore suggest that these motions are relevant to catalysis, because they involve all of the residues that make up the active site of the enzyme. Also, the correlated motions in the free enzyme show up only slightly when the substrate is in the *cis* configuration. The correlated motions become intense when the substrate is in the transition state configuration and are not present when the substrate is in the *trans* configuration. Therefore, these motions are coupled with the catalytic process in moving the substrate across the transition state from *cis* to *trans* and vice versa.

CONCLUDING REMARKS

In this study, we have shown that phosphorylation is an important mechanism by which transient conformational changes can be induced in proteins via post-translational modification of serine or threonine. The phosphate moiety and perturbation of the equilibrium distribution of protein conformations control the recognition mechanism with other proteins, such as human Pin1, and are central to regulating many intricate subcellular processes. Our results suggest that Pin1 recognizes the specific backbone conformation of its substrates using conformational selection and has a wider range of conformations that recognize the transition state. These different ensembles of conformations of Pin1 can be separately targeted with slightly different classes of compounds as a drug design strategy, exploiting the conformational variability of Pin1. Also, correlated motions in the free Pin1 enzyme are present in the ensemble of conformations that bind the substrate in the transition state configuration. The exact role of these dynamical coupled motions in the catalytic activity of Pin1 is yet to be determined and requires much further study. Fully understanding the atomistic basis for Pin1 recognition and the mechanism of catalysis of the *cis*−*trans* isomerization of the pSer/pThr-Pro motifs in signaling proteins will provide the foundation for the development of new classes of compounds for the treatment of many diseases, including cancer and Alzheimer's.

ASSOCIATED CONTENT

Supporting Information

Six figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

MD, molecular dynamics; aMD, accelerated molecular dynamics; FKBP, FK binding proteins; HIV, human immunodeficiency virus; PPIase, peptidyl prolyl *cis*−*trans* isomerase; Itk, interleukin tyrosine kinase; pSer, phosphorylated serine; pThr, phosphorylated threonine; SH2, Src homology 2; 5-HT3, 5-hydroxytryptamine-type 3 receptor.

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