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Structure and dynamics of Pin1 during catalysis by NMR

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Abstract

The link between internal enzyme motions and catalysis is poorly understood. Correlated motions in the us-ms timescale may be critical for enzyme function. We have characterized the backbone dynamics of the peptidyl-prolyl isomerase Pin1 catalytic domain in the free state and during catalysis. Pin1 is a prolyl isomerase of the parvulin family and specifically catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds. Pin1 has been shown to be essential for cell-cycle progression and to interact with the neuronal tau protein inhibiting its aggregation into fibrillar tangles as found in Alzheimer's disease. ¹⁵N relaxation dispersion measurements performed on Pin1 during catalysis reveal conformational exchange processes in the microsecond timescale. A subset of active site residues undergo kinetically similar exchange processes even in the absence of substrate, suggesting that this area is already "primed" for catalysis. Furthermore, structural data of the turning-over enzyme were obtained through inter- and intra-molecular NOEs. This analysis together with a characterization of the substrate concentration dependence of the conformational exchange allowed distinguishing of regions of the enzyme active site that are affected primarily by substrate binding versus substrate isomerization. Together these data suggest a model for the reaction trajectory of Pin1 catalysis.

Keywords

protein dynamics; NMR spectroscopy; peptidylprolyl isomerase; catalysis

Introduction

The mechanisms of protein function can be understood in terms of the protein's 3-dimensional structure and its variation over time (dynamics). After decades of research into the three-dimensional structures of proteins, a general framework for understanding structure-function relationships has appeared. This picture, however, is necessarily incomplete without an accurate description of protein dynamics[1]. In particular, the contribution of these conformational transitions to enzyme catalysis is poorly understood and, in most cases, has not been determined.

NMR spectroscopy has emerged as a powerful method in investigating the dynamics of biomolecules at atomic resolution across a wide range of timescales. Recently much attention has been focused into characterizing protein dynamics in the microsecond to millisecond

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timescales[2–23, and references therein]. These rates (10^2 – 10^6sec^{-1}) coincide with the rates for many biological processes such as ligand binding, protein folding and enzyme catalysis. Thus, being able to monitor conformational changes in this time regime may reveal functionally relevant internal motions of biomolecules.

The NMR experiments used to detect conformational exchange in the microsecond to millisecond timescale take advantage of the effect of this exchange on the observed transverse relaxation rate ($R2_{obs}$) of nuclei that are interconverting between states with different chemical shifts[24,25]. Conformational exchange causes an increase in $R2_{obs}$, called R_{ex} , leading to line broadening. Palmer and coworkers[3,26] have extended the method of relaxation dispersion allowing the determination of both kinetic and thermodynamic parameters of conformational exchange processes in proteins. This method explores the dependence of R_{ex} on the strength of a refocusing field according to the following expression (for a 2-state process in the fast-exchange limit)[27,28]:

$$R2_{obs} = R2_o + R_{ex} \quad (1)$$

$$R_{ex} = p_A p_B \frac{\Delta\omega^2}{k_{ex}} \left(1 - \frac{4\nu_{CPMG}}{k_{ex}} \tanh \frac{k_{ex}}{4\nu_{CPMG}} \right) \quad (2)$$

where p_A and p_B are the relative populations of the interconverting conformations, $\Delta\omega$ is the difference in chemical shift between the conformations, ν_{CPMG} is the strength of the refocusing field and k_{ex} is the sum of the rates of interconversion ($k_{forward} + k_{reverse}$).

Prolyl isomerases (PPIases) catalyze the cis-trans isomerization about prolyl peptide bonds [29]. Pin1 is unique among prolyl isomerases in that it specifically isomerizes phosphorylated Ser/Thr-Pro (pSer/pThr-Pro) bonds[30,31]. The acceleration of this isomerization may serve as a second regulatory mechanism in addition to phosphorylation in the form of a kinetic control [32,33]. Known substrates of Pin1 include a subset of cell cycle proteins (e.g. NIMA, Cdc25, Cyclin D) as well as the Alzheimer protein Tau and the transcriptional activator p53; however, the role of a conformational switch in these substrates and the mechanism of its regulation by Pin1 is not understood[34,35].

Pin1 is also distinguished by virtue of being composed by two domains: a WW domain and a catalytic domain. Both domains recognize pSer/pThr-Pro bonds and have been shown to be largely independent (both in terms of interaction and function) in solution[36,32]. Solution structures of Pin1 in the free state or bound to a sulfate ion[36] as well as an X-ray structure of the Pin1 catalytic domain (Pin1cat) in complex with an Ala-Pro dipeptide and a sulfate ion in the active site[37] have been solved. However, there are no available structures of an enzyme-substrate complex. Consequently, mechanistic interpretation is speculative. Pin1's catalytic mechanism is thought to involve an exposed cysteine residue attacking the carbonyl group of the pSer/pThr residue followed by rotation about the substrate prolyl bond. Neither the details of this mechanism, nor the contribution by other factors (e.g. conformational strain) are understood.

Here we perform structural and dynamic NMR experiments on Pin1cat during catalysis of a true substrate. Intermolecular NOE data collected during turnover of the peptide substrate together with backbone dynamics measured during catalysis reveal insight into the reaction trajectory. Conformational exchange in the free enzyme similar to that observed during turnover suggests the pre-existence of dynamics.

Results and Discussion

Titration of Pin1cat with the substrate WFYpSPRLKK

The minimal reaction scheme for a peptidyl-prolyl-cis/trans isomerase catalyzed reaction is given in Fig. 1C, in which the free enzyme (E) most likely comprises several substates. In order to distinguish the substrate binding step from the cis/trans isomerization step on the enzyme, a titration of Pin1cat with the substrate WFYpSPRLKK was performed. The activity of Pin1cat was verified before the titration via a protease-coupled isomer-specific assay[38] using a suc-AEPF-pNA peptide substrate. Pin1cat has a k_{cat}/K_m of $380 \pm 20 \text{ mM}^{-1} \text{ sec}^{-1}$ toward the assay substrate at 10°C .

Monitoring the amide resonances of Pin1cat as a function of substrate concentration (up to 15mM), we observe chemical shift changes in 18 residues, all localized to the active site of the enzyme. A subset of these are plotted in Figure 1A. All backbone amides of Pin1cat were present as a single peak at every point of the titration. Under these conditions of fast exchange we can calculate the dissociation constant K_d by fitting the chemical shift changes to a quadratic equation:

$$[complex] = \frac{[pep]_{tot} + [prot]_{tot} + K_d - \sqrt{([pep]_{tot} + [prot]_{tot} + K_d)^2 + 4[prot]_{tot}[pep]_{tot}}}{2} \quad (3)$$

where $[complex]$ can be calculated from:

$$\Delta\delta = \frac{[complex](\delta_{max})}{[prot]_{tot}} \quad (4)$$

where $\Delta\delta$ is the change in chemical shift at a particular substrate concentration and δ_{max} is the fitted difference in chemical shift between free and substrate-saturated enzyme. Fitting the individual titration data to equations 3 and 4, a K_d of $0.8 \text{ mM} \pm 0.15$ (mean $\pm$ std. dev.) was determined. From this K_d , a 95% saturation of Pin1cat is calculated at a substrate concentration of 15mM. With this information in hand, we were able to design experimental conditions to dissect the binding step from the isomerization step (see below).

Structural characterization of the turning-over complex

In order to obtain structural information on the turning-over complex of Pin1cat with the catalyzed substrate WFYpSPRLKK, we collected intra- and inter- molecular $^{13}\text{C}/^{15}\text{N}$ -edited 3D NOESY spectra in the substrate-saturated state. Intermolecular NOEs detected between the labelled protein and the unlabelled peptide can then be used to model a structure of the complex [39,40]. Importantly, the measured chemical shifts and NOEs are averaged over all the states that occur during catalysis since the sample represents a constantly turning-over enzyme.

A cursory analysis of the NOE data already suggests features of the action of Pin1cat. The majority of intermolecular NOEs between the substrate peptide and the enzyme correspond to substrate residues C-terminal to the prolyl bond, while signals from the N-terminus of the substrate are exchange broadened, indicative of a possible reorientation of residues N-terminal to the substrate proline due to the isomerization of the substrate. To obtain a coarse model of the complex structure, we started from the crystal structure of Pin1cat[37] and an extended substrate peptide with all peptide bonds in the trans conformation. Distance constraints based on the intermolecular NOEs (Table 1) were used for an initial energy minimization of the peptide-enzyme complex using the CHARMM[41] forcefields. We also used the position of

the sulfate ion in the crystal structure as a distance constraint for the phosphate moiety of the peptide (Table 1). The minimized complex was solvated in a shell of TIP3[42] waters 16 Å thick and the solvated system was equilibrated for 500ps at 300K after a 50ps heating step. The equilibrated complex has a $RMSD_{backbone}$ of 0.875 Å from the original minimization. A further minimization with no constraints was applied to the equilibrated complex resulting in the model shown in Figure 2. It has a $RMSD_{backbone}$ of 0.471 Å from the equilibrated structure.

In terms of protein conformation the model of the complex presents only minor differences when compared with the starting structure determined by Ranganathan et al[37]. In the resulting model the peptide substrate is bound in a cis conformation after minimization. Since we started with the trans conformation of the peptide before energy minimization, it suggests that at least some of the distance constraints derived from NOESY data represent the cis-bound conformation. However, we cannot rule out that this bias originates from the choice of starting structure of the protein in which the Ala-Pro peptide is bound in the cis conformation[37].

We want to emphasize that only a coarse model could be obtained because of the lack of inter- and intramolecular NOEs due to exchange broadening. However, the fact that a number of intermolecular NOEs are detected between the protein and peptide residues C-terminal to proline but no NOEs are detected for the N-terminal peptide residues suggests rearrangements primarily N-terminal to the proline.

Dissection of protein dynamics corresponding to binding and isomerization

Here, we investigated the dynamics of Pin1cat as a function of substrate. Transverse relaxation rates ($R2_{obs}$) of Pin1cat were measured at different levels of saturation by the peptide substrate. We measured these rates using a pulse program that minimizes the suppression of R_{ex} by using a single inversion pulse instead of the traditional train of refocusing pulses during the T2 delay [21] thus allowing us to detect the full increase in $R2_{obs}$ due to R_{ex} . In the free enzyme, surface loops reveal flexibility in the micro-to-millisecond time regime as detected by increased $R2_{obs}$. Upon addition of the substrate peptide, $R2_{obs}$ increases in residues surrounding the active site (Fig 3). However, different dependences are observed. A number of probes reveal a steep increase in $R2_{obs}$ reaching a maximum at a substrate concentration close to the dissociation constant (Fig. 3A). Beyond this point, the $R2_{obs}$ values for these residues show a decline and in the substrate saturated enzyme complex, $R2_{obs}$ for these residues is very close to that in the free enzyme. In contrast, for a second subset of probes, $R2_{obs}$ increases steadily to reach a plateau at saturating substrate concentrations (Fig. 3B).

These distinct trends indicate that different probes in the enzyme are reporting on distinct conformational exchange processes. The minimal model for the isomerase reaction predicts two different kinds of processes (see Fig. 1C): substrate binding-release and substrate isomerization (the chemical step). Probes with a maximum $R2_{obs}$ in the saturated state must reveal conformational changes related to substrate isomerization while probes that show a lower $R2_{obs}$ in the saturated state relative to a state where significant populations of free and bound enzyme are present are reporting mainly conformational changes associated with substrate binding[43,4]. Strikingly, the two sets of probes are localized in different regions of the active site (Fig. 3C). Probes with a maximum $R2_{obs}$ at the dissociation constant and minima in the free and saturated state are located C-terminal to the substrate prolyl bond that is isomerized in the reaction. In contrast, probes with a maximum $R2_{obs}$ in the saturated state surround the N-terminus of the substrate peptide up to the isomerized bond. This distribution suggests a reaction trajectory in which Pin1cat isomerizes the substrate peptide by rotating the peptide N-terminal to the prolyl bond while keeping the C-terminal portion of the substrate relatively fixed. This is, in turn, consistent with the structural data described above since almost no intermolecular NOEs from the N-terminal substrate residues are detected, presumably due

to their signals being exchange broadened by the cis/trans isomerization of the substrate when bound to the enzyme.

We performed a fit of R_{2obs} values as determined by the above experiment to a numerical simulation of the magnetization evolution of a three-state system described by the Bloch-McConnell equations[44] using the K_d determined from the substrate titration and k_{ex} as determined from the relaxation experiments discussed below. A grid search for the other parameters in the model gave multiple solutions, confirming that the 3-site model can be fit with this values but is under-determined.

Determination of the rate of enzyme motions associated with the catalytic step

The timescale of an exchange process can be estimated by comparing R_{ex} at different static (B_0) fields. This timescale is characterized by a parameter α given by[45]:

$$\alpha = \frac{(B_0 2 + B_0 1)(R_{ex} 2 - R_{ex} 1)}{(B_0 2 - B_0 1)(R_{ex} 2 + R_{ex} 1)} \quad (5)$$

where α takes values between 0 and 1 in a slow exchange process and values between 1 and 2 for an intermediate to fast process. Analysis of the dependence of R_{ex} on B_0 field showed that for all residues the exchange process in Pin1cat fell within the intermediate to fast time regime (Table 2). In this time regime, the relative populations and chemical shift differences of the states cannot be determined independently. Therefore, only kinetic parameters of the process can be extracted[45,46].

Using CPMG relaxation dispersion experiments (Fig 4A) we were able to detect and quantify the conformational exchange for residues in the active site of Pin1cat. As can be seen in Fig. 4, we can measure dynamics in probes surrounding the entirety of the substrate peptide, although the amplitude of the dispersion curve (an indicator of total R_{ex}) is largest for those residues surrounding the N-terminus of the peptide substrate. This is consistent with the above described results of the substrate-concentration dependence of R_{2obs} ; residues reporting on the chemical step have large amplitudes in the dispersion curves collected at substrate-saturated concentrations. We can still detect conformational exchange in the residues that are primarily sensitive to binding, but the magnitude of R_{ex} in these residues in the saturated complex is minimal compared to the magnitude of R_{2obs} observed in these residues at lower substrate concentrations (Figure 2A). The K_d of 0.8 mM and the fact that binding/dissociation is fast on the NMR time scale at all substrate concentrations ($k_{ex} \gg \Delta\omega$) implies that substrate binding and release at saturating concentrations is too fast to significantly contribute to the measured R_{ex} : k_{off} has to be 10^3 sec^{-1} or faster to be fast on the NMR timescale with $\Delta\omega$ values of about 100Hz, which means that k_{on} has to be at least $10^6 \text{ M}^{-1} \text{ sec}^{-1}$ resulting in a pseudo first order on-rate of at least $1.5 \times 10^4 \text{ sec}^{-1}$ at 15mM substrate and a k_{ex} for binding/dissociation of at least $1.6 \times 10^4 \text{ sec}^{-1}$. Moreover, our titration data (Fig 3) clearly show that R_{ex} due to binding at 15mM peptide concentration is less than 1Hz even for residues with an R_{ex} contribution of 40Hz at intermediate substrate concentrations (Fig 3A).

Most exchanging residues exhibited similar kinetic parameters when fit individually to the general Carver-Richards equation[27,47]. Individually, all but three residues exhibit a k_{ex} in the 900–2200 sec^{-1} range. Residues 75,76 and 77 exhibit much faster k_{ex} . We grouped the exchanging residues accordingly (Table 3) and tested a global fit by comparing the improvement in Chi-squared over the individual fits. Assignment to groups was confirmed with a jackknife analysis. Residues in group A fit together with a k_{ex} of 1200 $\pm$ 200 sec^{-1} (mean $\pm$ std. dev.). Residues 75–77 (Group B) fit together with a k_{ex} of 5000 $\pm$ 2000

sec^{-1} . This large uncertainty belies the fact that this faster rate is close to the limit that can be reliably measured by dispersion.

To rule out that this apparent faster rate was not an artifact of the low R_{ex} of these residues, we collected CPMG data at a second, lower temperature of 10°C. Dispersion profiles at this lower temperature confirmed that residues in Group B described a faster exchange process than those in Group A (Figure 6).

Dynamics in the free enzyme

Since NMR dynamics studies on a different prolyl-isomerase (CypA) in the free enzyme and during catalysis suggested that the motions necessary for catalysis are an intrinsic property of the enzyme[48], we wanted to test whether this might be a general property of prolyl isomerases. Therefore we examined the microsecond–millisecond dynamics of the free Pin1cat by CPMG dispersion experiments (Fig 5A). We observed dynamics in a number of probes within the large loop (residues 63–80) that contains the phosphate-binding residues as well as in part of the alpha-helix in the active site that contains the proposed catalytic cysteine 113 (see Fig. 5B). We observed the same distribution of two sets of exchanging residues as in the turning-over complex though with slightly different rates. The dispersion profiles can be globally fit with an exchange rate of $1500 \pm 300 \text{ sec}^{-1}$ for Group A and $3500 \pm 1500 \text{ sec}^{-1}$ for Group B at 25°C. The slightly larger uncertainty in the fitted k_{ex} of free Pin1cat compared to the fitted parameter in the complex data is mainly a consequence of the small R_{ex} amplitude (relatively small chemical shift differences and/or highly skewed populations, see equation 2) described by the dispersion curves of the free enzyme.

We note that for both the enzyme during turnover and the free enzyme, more complex models than a 2-site model are certainly possible, but the relaxation data can be well fit to this simple model. The presented data indicates that motions already exist in the free enzyme in regions where catalysis happens in the presence of substrate. The small amplitude of the dispersion curves together with the fact that the exchange is in the fast exchange regime for all residues prohibits extraction of $\Delta\omega$ values in the free enzyme. Moreover, all residues with R_{ex} in the turning-over enzyme are close to the substrate leading to direct chemical shift perturbations. Consequently, conclusions about the nature of the conformational transitions in the free enzyme relative to the motions during catalysis as performed in the dynamics studies of CypA [48] and Adk[49] cannot be made.

Conclusion

The conformational flexibility of proteins has been shown to be necessary for their function. Its role in enzyme catalysis, however, is not clear[1]. A detailed characterization of both conformational substates and dynamics will result in a more complete picture of how the structure and dynamics of the enzyme enable the catalysis of the chemical reaction. We have taken the first step in characterizing the dynamics of Pin1cat during substrate turnover. We have located the regions of the enzyme active site that undergo conformational changes upon substrate binding and during the catalytic step. The combination of NOE data collected during catalysis, R2 relaxation data as a function of substrate concentration and protein backbone dynamics data during catalysis point to a mechanism in which the substrate is anchored to the enzyme through peptide residues C-terminal to the proline and isomerization happens in regions N-terminal to proline concomitant with conformational changes in the enzyme. The rate of global conformational exchange detected during catalysis ($1200 \pm 200 \text{ sec}^{-1}$ at 25°C) is in the same time regime as turnover rates determined by the coupled chymotrypsin assay (3300 sec^{-1} at 10°C for the substrate AApSerPR[50]). The k_{cat} for the peptide used in our study is not known. For three residues (75–77) in a loop, a faster process is detected.

An exchange process in the active site in the free enzyme is detected with similar rates as measured during turnover. This phenomenon of micro- to millisecond motions in the free enzyme or when complexed with substrate analogues and the correspondance to turnover rates has been seen for a number of enzymes[2,5–7,9,¹¹–19,22,48,23]. Thus, an emerging view arises that protein motions relevant for enzyme catalysis are “built into” the conformational flexibility of the protein. Future work aims to modulate the flexibility of the protein and determine the effect on its activity.

Experimental Methods

Sample Preparation

The DNA sequence corresponding to the catalytic domain of Pin1 (residues 51–163) was cloned into the BamH/Not1 sites of the pGEX vector to produce a GST-Pin1cat fusion protein with a thrombin cut site. This construct was expressed in E.Coli BL21 cells containing a pACYC plasmid. For ¹⁵N labelled protein samples, cells were grown in ¹⁵N minimal medium to an OD of 0.8 and induced with 1mM IPTG for 6 hours. For ¹⁵N, ¹³C labelled protein samples, cells were grown in ¹⁵N, ¹³C minimal medium containing 20% of ¹⁵N, ¹³C enriched media (Silantes) and induced with 1mM IPTG for 6 hours. After harvesting, cells were resuspended in buffer containing 10 mM Sodium phosphate pH 7.4, 150 mM Sodium Chloride, 1 mM DTT and sonicated at 4°C. The soluble supernatant was applied to a glutathione resin for 10 minutes and washed with resuspension buffer. Restriction grade thrombin (Novagen) (1 U per mg of fusion protein) was applied to the resin and the cleavage reaction was allowed to proceed for 12 hours at 4°C. Pin1cat-containing fractions were then collected and run through a Benzimidine sepharose column (Amersham) to deplete thrombin. The resulting sample was concentrated to 1 mM and exchanged into 5 mM Sodium Phosphate pH 6.7 containing 1 mM TCEP.

The substrate peptide nWFYpSPRLKKc was synthesized by Fmoc solid phase chemistry by the core facility at Boston Biomedical Research Institute and purified by reverse-phase chromatography in house. Concentrated peptide stock was prepared in 5 mM Sodium Phosphate with pH adjusted to 6.7.

Enzyme Activity

Activity of the Pin1cat samples used in this study was assayed via a protease-coupled isomer-specific assay[38] using a suc-AEPF-pNA peptide (Bachem) as a substrate. Assays were performed at 10°C in 5mM Sodium Phosphate pH 6.7 and 1mM DTT.

Resonance Assignments

Triple resonance experiments were collected on a Varian Inova 500 spectrometer. Assignments of Pin1cat were generated by using the following standard 3D NMR experiments: HNCACB [51], CBCA-(CO)NH[52], C(CO)NH[53,54], HCCH-TOCSY[55] and ¹⁵N HSQC-TOCSY [56] spectra of a ¹⁵N, ¹³C labelled sample of free enzyme. Assignments of the saturated Pin1cat were generated by following backbone amide resonances as substrate peptide was added. HNCA[57], C(CO)NH and ¹⁵N HSQC-TOCSY spectra were used to confirm the assignments of the enzyme in the substrate-saturated state.

Assignments of the substrate peptide resonances were done through homonuclear TOCSY and ROESY spectra of a 2 mM peptide sample.

Substrate Titration

A 15:1 peptide:Pin1cat ratio was sufficient for a 95% enzyme saturation as confirmed by fitting the chemical shift data to a quadratic binding isotherm equation (see text). Difference in chemical shift (in ppm), $\Delta\delta$ for the titration was calculated as:

$$\Delta\delta = \sqrt{(\Delta H_N)^2 + (0.154 * \Delta N)^2} \quad (6)$$

Modeling of the Pin1cat-peptide complex

Inter- and intra-molecular NOEs were detected simultaneously and proton signals from the ^{15}N , ^{13}C labelled protein were selectively inverted[58,59]. A spectrum of inter-molecular NOEs between the unlabelled substrate and the isotopically labelled protein is thus generated and used to define a set of distance constraints for the complex. A structure model was constructed based on the previously published crystal structure of Pin1cat[37] and a generated extended structure of the substrate peptide. The model was then constrained in vacuum using the obtained distance constraints in Table 1. Energy minimization was then performed in two stages (steepest descent followed by 10000 steps of conjugate gradient). The minimized complex was then solvated with a shell of TIP3 waters[42] using Helmut Grubmueller's SOLVATE program and equilibrated for 500ps before being minimized again without constraints. All energy minimization and molecular dynamics simulations were run with CHARMM[41].

NMR Relaxation Experiments

Two kinds of NMR relaxation measurements were taken on ^{15}N labelled Pin1cat (1 mM in 5 mM Sodium phosphate, pH 6.7; 1 mM DTT) on a Varian Inova 600 spectrometer at 25°C. First, R_{2obs} was measured as a function of substrate concentration using a pulse program that employs a single inversion pulse during the T2 delay to minimize suppression of R_{ex} [21]. Spectra were collected on the free enzyme and of the enzyme in the presence of substrate peptide at increasing concentrations ranging from 0.1 mM to 15 mM. For each substrate:enzyme ratio, a series of spectra with T2 delays ranging from 5 msec to 120 msec were collected. Crosspeak intensities were fitted to a single exponential decay to obtain R_{2obs} values. For higher substrate:enzyme ratio samples, an offset in R_{2obs} due to increased sample viscosity was estimated from the trimmed mean values. R_{2obs} values as determined by the above experiment were fit to a numerical simulation of the magnetization evolution of a three-state system described by the Bloch-McConnell equations[44] calculated using Mathematica.[43]

For quantification of the rates of chemical exchange, we used a ^{15}N CPMG-TROSY pulse sequence.[28,26,60]. In this experiment the effective transverse relaxation rate ($R_{2obs} = R_{2o} + R_{ex}$) is measured as a function of the strength of an B1 field applied through a train of 180° pulses during the T2 delay. Samples consisted of 1mM Pin1cat (with 15mM substrate peptide for the saturated enzyme experiments) in 5mM sodium phosphate, pH 6.7, 1mM DTT. The dispersion curves were collected with a T2 delay of 60 ms with a total of 17 field strengths ranging from 33 to 1000 Hz in an interleaved manner. Data were acquired on Varian Inova 500 and 600 spectrometers for the free enzyme and on Varian Inova 500 and 720 spectrometers for the saturated enzyme. Data at a lower temperature for both the free enzyme and the 95% saturated complex were collected at 600MHz with a relaxation delay of 50ms. R_{2obs} at each CPMG field strength was extracted as described previously[28] except uncertainty in the measured R_{2obs} was estimated from the average variance in R_{2obs} observed in non-exchanging residues for each dataset. This uncertainty takes into account difference in signal/noise ratios

as well as systematic errors[46]. This approach resulted in a more robust error estimation than by relying on the estimate based on a small number of duplicate measurements.

Kinetic parameters were estimated by fitting the dispersion curves to a 2-state model of the general Carver-Richards equation[27,47]. Separation of exchanging residues into groups was verified by comparing Chi-squared probabilities between individual and global fits. Proper assignment to groups were confirmed by jackknife analysis. Kinetic parameters and their uncertainties are calculated via a Monte-Carlo approach. In this approach 50 datasets of each residue are generated based on an initial fit of the collected data to which gaussian noise based on the experimental uncertainties of $R2_{obs}$ are added. From the generated data sets 50 global fits are performed. The reported kinetic parameter is the mean and its uncertainty is the standard deviation (see text).

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Figure 1.

(A) Titration of Pin1 catalytic domain with substrate peptide WFYsPRLKK. Subset of Pin1 cat residues exhibiting changes in chemical shift as a function of substrate concentration are plotted. Chemical shift changes ($\Delta\delta$) are combined $^1\text{H}/^{15}\text{N}$ chemical shift changes calculated according to equation 6.

K_d for the complex was determined to be $0.80\text{mM} \pm 0.15$. Residues plotted are Gln75 (▲), Gly123 (▼), Gly128 (◆), Gln129 (○) and Gly155 (△). (B) Reaction catalyzed by Pin1. (C) Minimal reaction scheme for the reaction catalyzed by Pin1. The reversible reaction allows for measurement of the isomerization step when the enzyme is saturated.

Figure 2.

Model of the structure of the Pin1 catalytic domain in complex with the substrate peptide WFYpSPRLKK. Pin1cat is shown in gold with the sidechains of enzyme residues involved distance restraints depicted in orange. The substrate peptide is shown in green with the pS-Pro residues highlighted in cyan. The starting structure used was Pin1cat (PDB: 1PIN[37]) after removal of the Ala-Pro dipeptide and the sulfate ion in the active site. Distance constraints (shown as black lines) were defined based on intermolecular NOEs measured during catalytic turnover and on distances to the sulfate ion in the starting structure. Complex was energy-minimised and equilibrated using CHARMM[41].

Figure 3.

Conformational exchange of Pin1cat during catalysis. Transverse relaxation rates ($R2_{obs}$) of Pin1cat were measured as a function of substrate concentration. Two distinct trends can be clearly observed: (A) The contribution to $R2_{obs}$ from conformational exchange reaches a maximum as the substrate concentration approaches the dissociation constant before diminishing close to zero in the saturated enzyme. Probes describing this trend are shown in blue in the complex structure (C); the curve shows the individual measurements for Gly128 (A). (B) A second set of probes has a maximum conformational exchange contribution as the enzyme is saturated with substrate peptide, shown in red in (C). The curve shows the measurements corresponding to Gly155. Curves in A and B were fit by explicit numerical simulation of Bloch-McConnell equations[44] of magnetization evolution of a 3-state model (see Fig. 1C) using k_{ex} as calculated from CPMG experiments and K_d as calculated from the substrate titration (see text).

Figure 4.

(A) Quantitative analysis of exchange dynamics in Pin1cat during turnover. Dependence of $R_{2,obs}$ on a CPMG field was measured in the presence of saturating concentrations of substrate. Relaxation dispersion curves are shown for a subset of residues at 16.9T (Top) and 11.7T (Bottom) for residues in group A. A global fit to a 2-site exchange model reports an exchange rate of $1200 \pm 200 \text{ sec}^{-1}$ for residues in Group A and a rate of $5000 \pm 2000 \text{ sec}^{-1}$ is reported by residues in Group B at 25°C. Plots are shown for residues Arg69 (○), Ser114 (Δ), Gly155 (■) and Gln129 (◆). (B) Backbone ¹⁵N probes for Group A (Red) and Group B (Purple) are plotted on the structure.

Figure 5.

(A) Quantitative analysis of exchange dynamics in free Pin1cat measured at 14.1T (Top) and 11.7T (Bottom). Residues in Groups A and B can be globally fit to a 2-site model with an exchange rate of $1700 \pm 500 \text{ sec}^{-1}$ and a rate of $3500 \pm 1500 \text{ sec}^{-1}$ at 25°C , respectively. Plots are shown for residues Arg69 ($\circ$), Ser114 (Δ), Gln75 ($\blacksquare$) and Lys77 ($\blacklozenge$). (B) Residues in group A are highlighted in red while those in Group B are highlighted in purple. These dynamic regions of the free enzyme are a subset of the dynamic regions observed in the active complex (Fig. 4).

Figure 6.

Quantitative analysis of exchange dynamics in Pin1cat at 10°C. At a lower temperature, the two groups of exchanging residues can be more accurately detected. Top: Group B residues (Lys77($\diamond$), Glu76($\blacksquare$) and Gln75($\circ$)) in both the saturated complex (A) and the free enzyme (B, residues Lys77($\square$) and Gln75($\triangle$)) are in fast exchange (k_{ex} 4100 $\pm$ 1600 sec^{-1}). Bottom: this is clearly distinct from Group A residues which experience slower (k_{ex} of 1000 $\pm$ 300 sec^{-1}) conformational exchange in both the saturated complex (C, residues Ser114($\circ$), Gln129($\diamond$) and Ser155($\blacksquare$)) and the free enzyme (D, residues Arg69(∇) and Ser114($\bullet$)). Group B residues are located in a single flexible loop. (See figs 4 and 5).

Table 1

List of Intermolecular Distance Restraints

Distance Restraints from Intermolecular NOEs ¹			
Pin1cat residue	¹ H(Pin1cat)	Peptide residue	¹ H(Peptide)
Ala116	HB	Tyr3	HD2
Leu61	HD1	pSer4	HB1
Asp153	HB1	pSer4	HA
Val55	HG1	Pro5	HG1
Pro133	HD1	Pro5	HG2
Lys117	HA	Leu7	HD1
Arg119	HG2	Leu7	HD1
Distance Restraints from Bound Sulfate in PDB (1PIN) ²			
Pin1cat residue	Atom (Pin1cat)	Peptide residue	Atom (Peptide)
Arg68	CZ	pSer4	P1
Arg69	CZ	pSer4	P1

¹ Allowed distance range was 1.8–6.0 Å

² Allowed distance range was 1.8–8.0 Å

Table 2Scaling factors α for Pin1cat.

Free Enzyme.		
Pin1cat residue²	α^3	+/-⁴
Gln75	1.95	0.62
Glu76	1.95	0.46
Lys77	1.95	0.62
Enzyme-Substrate Complex⁵		
Pin1cat residue²	α^3	+/-⁴
Arg68	1.95	0.31
Arg69	1.95	0.29
Ser71	1.93	0.73
Ser72	1.93	0.56
Gln75	1.93	0.38
Lys77	1.94	0.90
Ser114	1.94	0.40
Ser115	1.95	0.35
Lys117	1.93	0.56
Gly123	1.94	0.34
Gly128	1.94	0.58
Gln129	1.94	0.53
Lys132	1.92	0.67
Ser154	1.94	0.61
Gly155	1.15	0.26
Ile158	1.94	0.60

¹Relaxation data collected at 11.7T and 14.1T² α values are reported for exchanging residues if the uncertainty in α is less than 50%³Values of R_{ex} used in calculating α were estimated from the fits of $R2(vCPMG)$ data to the general Carver-Richards equation as $R_{ex} = R2_{obs}(33Hz) - R2_o$ ⁴Uncertainties in α are calculated by propagating the uncertainties in the fitted $R2_o$ and $R2_{obs}(33Hz)$ ⁵Relaxation data collected at 11.7T and 16.9T

Table 3

Conformational Exchange in Pin1cat.

Residue	Complex ¹			Free enzyme ²		
	Group A	Group B	Group B	Group A	Group B	Group B
	$R_{ex}(sec^{-1})^3$	+/-	$R_{ex}(sec^{-1})$	+/-	$R_{ex}(sec^{-1})$	+/-
Ser67	1.4	0.28				
Arg68	11.5	0.64				
Arg69	14.31	0.73		2.07	0.42	
Ser71	2.92	0.38				
Ser72	3.27	0.33				
Gln75			10.77	0.72		3.66 0.37
Glu76			14.18	0.96		6.77 0.13
Lys77			7.18	0.76		3.35 0.53
Cys113	4.52	0.9		1.31	0.21	
Ser114	6.74	0.47		3.02	0.41	
Ser115	9.99	0.62		1.71	0.22	
Lys117	3.48	0.35				
Leu122	2.30	0.44				
Gly123	7.68	0.46				
Gly128	4.54	0.47				
Gln129	7.16	0.68				
Gln131	3.74	0.54				
Lys132	4.21	0.50				
Ser154	4.51	0.48				
Gly155	13.46	0.73				
Ile158	3.91	0.42				

¹ Relaxation data collected at 16.9T at 25C

² Relaxation data collected at 14.1T at 25C

³ Values of R_{ex} were calculated from the fits of $R2(\nu_{CPMG})$ data to the Carver-Richards equation in the fast-exchange regime.