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Kinetic Isotope Effects Support the Twisted Amide Mechanism of Pin1 Peptidyl-Prolyl Isomerase

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Abstract

The Pin1 peptidyl-prolyl isomerase (PPIase) catalyzes isomerization of pSer/pThr-Pro motifs in regulating the cell cycle. Peptide substrates, Ac–Phe–Phe–phosphoSer–Pro–Arg–*p*-nitroaniline, were synthesized in unlabeled form, and with deuterium labeled Ser-d_3 and Pro-d_7 amino acids. Kinetic data was collected as a function of Pin1 concentration to measure kinetic isotope effects (KIE) on catalytic efficiency (k_{cat}/K_m). The normal secondary (2°) KIE value measured for the Ser-d_3 substrate (k_H/k_D = 1.6 ± 0.2) indicates that the serine carbonyl does not rehybridize from sp^2 to sp^3 in the rate-determining step, ruling out a nucleophilic addition mechanism. The normal 2° KIE can be explained by hyperconjugation between Ser α-C–H/D and C=O, and release of steric strain upon rotation of the amide bond from cis to syn-exo. The inverse 2° KIE value (k_H/k_D = 0.86 ± 0.08) measured for the Pro-d_7 substrate indicates rehybridization of the prolyl nitrogen from sp^2 to sp^3 during the rate-limiting step of isomerization. No solvent kinetic isotope was measured by NMR exchange spectroscopy (EXSY) (k_{H2O}/k_{D2O} = 0.92 ± 0.12), indicating little or no involvement of exchangeable protons in the mechanism. These results support the formation of a simple twisted-amide transition state as the mechanism for peptidyl prolyl isomerization catalyzed by Pin1. A model of the reaction mechanism is presented using crystal structures of Pin1 with ground state analogues and an inhibitor that resembles a twisted amide transition state.

Keywords

Pin1; peptidyl-prolyl isomerase; PPIase mechanism; solvent deuterium kinetic isotope effects; EXSY; secondary kinetic isotope effects

Protein interacting with NIMA-1 (Pin1, NCBI accession #: AAC50492), a peptidyl-prolyl isomerase (PPIase), catalyzes the cis-trans isomerization of pSer/pThr–Pro substrates in vivo and in vitro.(1, 2) The details of the Pin1 catalytic mechanism of cis-trans isomerization have not been completely elucidated, although it is well understood that PPIases must operate by breaking the π-bond character of the amide C–N bond.(3, 4) Two basic mechanisms have been proposed for Pin1: (1) nucleophilic addition by an enzyme thiol at the carbonyl carbon,(5) or (2) twisting the amide bond out of conjugation, facilitated by

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Supporting Information Available. Schemes, experimental procedures for the synthesis, and NMR and MS characterization data for labeled and unlabeled compounds **1–11**, and statistical analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

hydrogen bonding to the prolyl nitrogen.(6, 7) Both of these represent ways to disrupt the carbon-nitrogen π-bond of the amide and leave it as only a single bond, which can rotate easily.

For human cyclophilin A (hCyPA), the nucleophilic catalysis mechanism was ruled out by mutagenesis,(8, 9) and a normal secondary deuterium kinetic isotope effect (KIE).(10, 11) For both hCyPA and FK506 binding protein (FKBP), general acid or base mechanisms were ruled out because the enzymatic reaction rate is independent of the pH between 5 and 9, and small, inverse solvent deuterium KIEs were measured.(10, 12) For Pin1 with substrate Ala-Glu-Pro-Phe-*p*NA, the rate was optimal between pH 6 to 7, and dependent upon an ionizable substrate Glu residue preceding Pro, yet pH independent with the corresponding Ala–Pro substrate.(5)

The twisted-amide mechanism for PPIases was first proposed based on the bound conformation of the FK506 α-ketoamide,(12, 13) as well as 2° KIEs.(10, 11) It has been suggested the PPIases bind the Xaa-Pro substrate and distort the amide bond by pyramidalizing the prolyl nitrogen through hydrogen bonding.(6, 10, 14, 15) Calculation of the FKBP reaction pathway showed substrate intramolecular donation of a hydrogen bond from the C-terminal amide N–H to the prolyl N in the transition state.(14) Non-enzymatic catalysis by intramolecular hydrogen bonding was demonstrated in solution.(16) Additional evidence for N–H participation is that isomerization of Pro amides is much faster than Pro esters.(17)

The energy for catalysis for FKBP comes mainly from substrate destabilization by twisting the carbonyl out of plane with nitrogen by as much as 24°.(14) Only a small amount of the energy was calculated to come from transition-state stabilization, 1.5 and 2.3 kcal/mol for cis and trans substrate respectively out of a total of 6.2 kcal/mol.(14) The X-ray structure of a tetrapeptide substrate bound to hCyPA showed Xaa-*cis*-Pro amide ω-bonds with angles of 45° and 22° for independent molecules, indicating destabilization of substrate upon binding.(18) The orientation of the substrate in the hCyPA active site does not permit an intramolecular hydrogen bond.(18) We proposed that the active site Arg55 in hCyPA could act as the hydrogen-bond donor on the basis of mutagenesis,(9, 15) supported by X-ray crystallography,(18, 19) and later by calculations on hCyPA.(20) All these results support a mechanism for FKBP and cyclophilin PPIases that is a combination of substrate distortion to a twisted amide, and transition state stabilization by hydrogen bonding to the transient proline nitrogen lone pair.

The proximity of the active site Cys113 to the Ala–Pro carbonyl in the first crystal structure of Pin1 led to a proposal of nucleophilic catalysis by Pin1.(5) Mutation of active site residues of Pin1 resulted in decreased, but not total, abrogation of activity. C113A had a 120-fold, and C113S had a 20- to 50-fold loss of activity.(5) Pin1 C113D was active in a yeast $Ess^{-/-}$ complementation assay.(21) H59Q, H157N, and H157L showed complementation, although H157A failed to complement in yeast.(21) Based on FK506 inhibition of FKBP, we designed and synthesized α-ketoamides to mimic the twisted amide transition state, or to accept nucleophilic addition of the Pin1 Cys113 thiol to the carbonyl, but the two α-ketoamides were poor Pin1 inhibitors.(22) Similarly, poor inhibition of Pin1 by cyclohexyl ketone inhibitors suggested that the nucleophilic addition mechanism with the Pin1 Cys113 thiol is unlikely.(23)

Our crystal structure of Pin1 with a reduced amide inhibitor bound in the active site showed that it resembled the proposed twisted amide transition state (Figure 1).(24) Although the structure shows the lack of an enzymatic hydrogen bond to the prolyl nitrogen, the enzyme Gln131 backbone NH hydrogen bonds to the inhibitor Pro C=O to position the tryptamine

αNH in close proximity (N–N distance 2.63Å) to the lone pair on the prolyl nitrogen (Figure 1).(24) Docking of cyclohexyl ketone inhibitors suggested that binding in the Pin1 active site distorts the substrate into a *trans*-pyrrolidine conformation (referring to the ring stereochemistry, and not to be confused with the *trans*-prolyl amide) to facilitate amide isomerization, which led us to propose that the substrate is destabilized in a stretched conformation upon binding to the Pin1 catalytic site.(23) Similarly, the structure of the complex of Pin1 with the reduced amide inhibitor shows a *trans*-pyrrolidine conformation with the substituents on the ring 0.5 Å farther apart than they would be in a *cis*-pyrrolidine conformation.(24) Recent calculations support a mechanism for Pin1 that includes substrate destabilization and a twisted amide conformation in the transition state.(25)

Herein, we measured substrate and solvent deuterium KIEs with substrates, **1 – 4** to further investigate the catalytic mechanism of Pin1.

Ac–Phe–Phe–pSer–Pro–Arg–*p*NA	**1**
Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA	**2**
Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA	**3**
$^{+}H_3N$–Glu–Gln–Pro–Leu–pThr–Pro–Val–Asp–Leu–O^{-}	**4**

EXPERIMENTAL PROCEDURES

Synthesis of Substrates

The synthesis and characterization of substrates **1–3** was performed according to Bernhardt et al., with modifications as described in the Supporting Information.(26) Substrate **4** was purchased from Anaspec, Inc.(27)

Pin1 kinetic isotope effect PPIase assay.(28)

The unlabeled substrate **1** was synthesized independently for the k_{cat}/K_m determinations for the KIE of the Ser-d_3 and Pro-d_7 substrates, **2** and **3**. Measurements for direct comparison were all taken on the same day. Substrate **1–3** concentrations were determined by UV absorbance at 390 nm, ε_{390} = 12,500 $M^{-1}{\cdot}cm^{-1}$.(28) The concentrations of the cis component of substrates **1–3** were determined by the UV-Vis absorbance difference at 390 nm after complete cleavage of the *p*-nitroaniline by trypsin after 3 min. Observed rate constants (k_{obs}) were measured at fixed concentrations of substrate: (1) for unlabeled **1**, final [cis] = 22 μM (44% cis), and Ser-d_3 **2** final [cis] = 13 μM (54% cis); and (2) for unlabeled **1**, final [cis] = 11 μM (54% cis), and Pro-d_7 **3** final [cis] = 8.3 μM (34% cis). HEPES buffer pH 7.0 (35 mM stock, 1.05 mL) and trypsin (60 mg/mL in 0.001 N HCl, 120 μL) were added to a 1.0 cm polypropylene cuvette, and pre-equilibrated to 4 °C. The thermal isomerization rate constant k_3 was determined in the absence of Pin1 at 4 °C. Pin1 in 20 mM Tris·HCl, pH 7.0 (200 nM stock at 4 °C, 10 μL) was added to final concentrations: 5.0, 7.0, 10, 12, 15, and 20 nM in duplicate for Ser-d_3, and 5.0, 7.5, 10, 12.5, 15, 20 and 22 nM in triplicate for Pro-d_7 KIE determinations. Substrate for experiment: (1) unlabeled 10 μL of 5.9 mM stock, and Ser-d_3 10 μL of 2.9 mM stock in dry 0.48 M LiCl/TFE; or (2) unlabeled 7.0 μL of 4.5 mM stock, and Pro-d_7 7.0 μL of 5.3 mM stock in dry 0.47 M LiCl/TFE, pre-equilibrated at 4°C, was added via syringe to a final volume of 1.2 mL, and the assay solutions were mixed vigorously by rapid inversion three times, with a time delay of 3 – 5 sec before UV monitoring. The progress of the reaction was monitored at 390 nm for 90 s in a dry cold room to prevent moisture condensation on the UV cuvette during reaction monitoring.

Kinetic analysis of Pin1 PPIase results

Pin1 kinetics were analyzed as described by Kofron et al. 1991.(29) The absorbance vs. rate data were analyzed by nonlinear least squares fit to an exponential equation, using TableCurve software v. 5.2 to measure k_{obs}.(29) Rate constants (k_{obs}) were plotted as a function of Pin1 concentrations. The catalytic efficiencies, k_{cat}/K_m, were calculated from the linear fit to the plot of k_{obs} *vs.* enzyme concentration [E], equation (1), where k_3 is the thermal background rate measured in the absence of Pin1 with each substrate.(29)

$$k_{\mathrm{obs}}=k_3+(k_{\mathrm{cat}}/K_{\mathrm{m}})[\mathrm{E}] \quad (1)$$

The fitted value of the slope was taken as k_{cat}/K_m, which was used to calculate kinetic isotope effect ratios. The statistical error analyses are given in Supporting Information.

Solvent deuterium kinetic isotope by NMR

Standard Pin1 NMR buffer was prepared with 30 mM imidazole-d_4, 30 mM NaCl, 0.03% NaN_3, and 10% D_2O in distilled, de-ionized H_2O. The pH was adjusted to 6.60 with HCl. Cdc25 phosphopeptide EQPLpTPVTDL **4**, purchased from Anaspec Inc, was supplied with HPLC and MS analysis (purity > 96%). Peptide **4** was weighed and dissolved in Pin1 NMR buffer. The pH of peptide stock was adjusted to a value of 6.6 by addition of dilute NaOH or NaOD solution. Aliquots of 55.8 μL of peptides at 12.5 mM were stored at −20 °C until use. Deuterated Pin1 NMR buffer was prepared with 30 mM imidazole-d_4, 30 mM NaCl, 0.03% NaN_3, and D_2O. pD was adjusted to 6.60 (pH reading of 6.20) using DCl. Solutions were filtered and degassed. Freshly purified wild-type Pin1 protein was split into two tubes. One buffer was exchanged with the protonated Pin1 NMR buffer, and the other was exchanged into the deuterated Pin1 NMR buffer at 4°C for 16 h. The protein concentration was calculated using a Bradford assay (BSA standard), and then diluted to a final concentration of 50 μM in a Shigemi tube along with 2 mM of pCdc25 peptide **4** and 5 mM DTT-d_{10}.

The 2-D ^{1}H-^{1}H exchange spectroscopy (EXSY) spectra were recorded using the standard NOESY-based pulse scheme,(30) supplemented by pThr methyl frequency-selective pulses to enhance resolution. The spectra were recorded at 295K at 16.4T (700 MHz ^{1}H Larmor frequency). The exchange mixing times were 5, 10, 25, 30, 40, 50 (×2), 75, 100 (×2), 200, 350, and 450 ms for the protonated sample, and 5, 10, 50 (×2), 75, 100, 150, 200, 225, 300, 350, and 450 (×2) ms for the deuterated sample. To estimate the net exchange rate constants, k_{EX}, we fitted the ratios of the cis-to-trans exchange cross-peaks over the trans diagonal peaks as a function of the mixing time t_{mix}, to equation (2).(30–32)

$$ratio(t_{mix})=\frac{\left(1-\mathrm{e}^{-k_{EX}t_{mix}}\right)k_{TC,OBS}}{k_{CT,OBS}+k_{TC,OBS}\mathrm{e}^{-k_{EX}t_{mix}}} \quad (2)$$

In equation (2), the adjustable parameters for fitting were $k_{TC,obs}$ and $k_{CT,obs}$ where $k_{EX} = k_{TC,obs} + k_{CT,obs}$. Uncertainties in the rate constants were estimated via Monte Carlo simulations based on the duplicate spectra. In a sample containing 2 mM of Cdc25 phosphopeptide **4** without Pin1, EXSY cross-peaks were absent because exchange was below the limit of detection.

RESULTS AND DISCUSSION

Unlabeled Ac–Phe–Phe–pSer–Pro–Arg–pNA **1**, and deuterium labeled substrates **2–3**, incorporating Ser-d_3, and Pro-d_7 amino acids, were synthesized for the investigation of the

Pin1 catalytic mechanism. The Ser-d_3 and Pro-d_7 substrates used include both α and β secondary kinetic isotopic effects. In the approach to the transition state, the C–H/D bonds near the reacting center of the substrate relax or tighten, resulting in normal or inverse KIEs. (33, 34)

Normal 2° KIE for Ser-d_3 substrate

In the mechanism first proposed for Pin1, the serine carbonyl was thought to undergo nucleophilic addition by the Pin1 Cys113 thiol, with rehybridization from sp^2 to sp^3, forming a tetrahedral transition state.(5) To test this hypothesis, we prepared substrate, Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2**, labeled at the α- and β-positions of Ser. For Ac–Phe–Phe–pSer–Pro–Arg–*p*NA the k_{cat}/K_m value of 0.00423 ± 0.00035 nM^{-1}-s^{-1} was measured. For Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, with deuterium labeling at both the α- and β-carbons of serine, the k_{cat}/K_m value of 0.00271 ± 0.00034 nM^{-1}-s^{-1} was measured. The ratio of k_{cat}/K_m for the unlabeled and labeled substrates gave a normal 2° KIE value of 1.6 ± 0.2 for duplicate data points at 7 Pin1 concentrations (Figure 2).

The normal 2° KIE indicates that the serine carbonyl does not rehybridize from sp^2 to sp^3 (Figure 2A), in agreement with the direction measured for a cyclophilin Cα deuterated substrate (k_H/k_D = 1.13 ± 0.01).(10) On the other hand, in amide bond *formation*, inverse 2° KIE effects were found, as expected for carbonyl rehybridization from sp^2 to sp^3.(35)

Hyperconjugation in the transition state most readily explains the α-2° KIE.(10) The αC–(H/D) σ-bond can overlap more easily with the ketone-like C=O π-bond in the transition state than with the amide C=O of the ground state, in which π-overlap with the prolyl nitrogen lone pair dominates (Figure 2B). Geometric and steric hindrance factors strongly influence β-2° KIEs. The larger magnitude 2° KIE observed for Pin1 than for cyclophilin is probably due to the β-CD_2 labeling of the Pin1 substrate, which indicates steric relief at this position of Ser in the twisted-amide transition state (Figure 3).(10, 34)

During the cis-trans isomerization step of the labeled substrate, the cis conformation begins in a ground-state conformation in which the β$C(H/D)_2$ bonds at serine sterically interact with the proline ring (Figure 2). In the transition state, the steric interaction is relieved, a loosening of the structure, which is more important for the C–H than the C–D substrate.(34) This steric interaction in the ground state is part of the destabilization of prolyl amides towards isomerization.(14, 20)

Inverse 2° KIE for Pro-d_7 substrate

We sought next to show that pyramidalization occurs at the prolyl amide nitrogen. For Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, a k_{cat}/K_m value of 0.00322 ± 0.00025 nM^{-1}-s^{-1} was measured. For Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA, with the proline ring fully labeled with deuterium, a k_{cat}/K_m value of 0.00376 ± 0.00021 nM^{-1}-s^{-1} was measured—isomerization of the Pro-d_7 labeled substrate occurred faster than the unlabeled analogue. The ratio of k_{cat}/K_m for the unlabeled and labeled substrates gave an inverse 2° KIE value of 0.86 ± 0.08 for triplicate data points (Figure 3A). An inverse KIE ($k_H/k_D < 1$) occurs when an sp^2 center becomes an sp^3 center in the transition state.(33, 34) The inverse the 2° KIE value indicates more steric strain in the transition state when the proline nitrogen rehybridizes from sp^2 to sp^3 (Figure 3B). The deuteriums the Pro-d_7 substrate are in close and rigid proximity to the rehybridizing prolyl amide nitrogen reaction center, facilitating the twisted-amide conformation in the prolyl amide bond, and increasing the rate of isomerization. An inverse 2° KIE has also been measured for uncatalyzed cis-trans isomerization in *N*-methylformamide,(36) but not prior to this for PPIases.

Solvent Kinetic Isotope Effects

Since trypsin, the coupling enzyme for the UV/Vis-based continuous assay, has a solvent deuterium kinetic isotope effect of 1.5 to 4, (37) it was difficult to add enough trypsin in D_2O to be sure that trypsin proteolysis was not rate-limiting, and without adding so much trypsin that Pin1 itself was proteolyzed at a significant rate. Thus, to investigate the enzymatic mechanism of Pin1 PPIase, the solvent deuterium isotope effect, k_{H2O}/k_{D2O}, was measured by 2D NMR EXSY (Figure 4). No change in the Pin1 PPIase rate in D_2O (k_{EX} = 28.5 ± 3.7 s^{-1}) was observed relative to H_2O (k_{EX} = 26.2 ± 1.0 s^{-1}), giving the ratio of k_{H2O}/k_{D2O} = 0.92 ± 0.12 (Figure 4). The difference in the observed rates at the plateaus of the build-up curves is likely due to a thermodynamic isotope effect (Figure 4). (31, 32)

The lack of a solvent deuterium isotope effect suggests that a possible hydrogen bond within the 5-membered ring between the tryptamine Xaa-NH and the Pro N in the transition state is too strained to show a significant deuterium isotope effect (Figure 5). In our crystal structure of a reduced amide (tertiary amine) inhibitor bound to Pin1, there was no crystallographic water found in this region to bridge between the NH and the Pro N.(24) The proline nitrogen is pyramidalized with the lone pair or proton directed away from potential hydrogen bond donors in the active site, and toward the inside of the inhibitor β-turn-like conformation (Figure 1).(24) This twisted amide conformation, with the carbonyl oxygen directed towards the enzyme on the same side as the nitrogen alkyl substituents, the syn-exo conformation, is the low energy transition state for amide bond rotation, and the low energy pathway calculated by quantum mechanics/molecular mechanics (QM/MM) for Pin1.(25, 38) This conformation positions the incipient lone pair on the Pro nitrogen to form a hydrogen bond in the transition state with the NH_{i+1} of the residue after Pro (Figure 1). The NH is, in turn, positioned by a hydrogen bond between the Pro C=O and the NH of Gln131 in Pin1 (Figure 2).(7, 24, 39) Even though the distance between the two substrate nitrogens is 2.63 Å, the N–H--N angle in such a 5-membered ring would allow only a strained hydrogen bond.(40, 41)

Such a weak hydrogen bond suggests no difference from the mechanism of amide rotation catalysis calculated for FKBP and hCyPA.(14, 20) For these PPIases, the solvent deuterium KIEs were inverse: 0.92 and 0.98 respectively,(10, 11) indicative of poor hydrogen bonding angles in a 5-membered ring, or weaker hydrogen bonding upon approach to the transition state. The lack of a solvent deuterium isotope effect value for Pin1 suggests that the prolyl amide nitrogen is not strongly hydrogen bonded in the rate-determining step (Figure 1).

Taken together with previously published results, we propose a mechanism for Pin1 that includes a strained hydrogen bond from the Pro–Xaa amide NH to the proline nitrogen in a twisted amide transition state (Figure 5). Strained hydrogen bonds are involved in a number of other enzymatic reaction mechanisms.(40–42) Our (*E*)- and (*Z*)-alkene ground state analogues, and reduced amide inhibitor-Pin1 complex structures give snapshots of the proposed reaction pathway for Pin1 (Figure 5).(24, 39) There is no need to invoke large arm swinging motions, rather minimal atomic motion can explain the mechanism.(43) Indeed, since the phosphate group and Pro carbonyl are locked in place by hydrogen bonding in both ground state analogue and reduced amide complex structures, very little motion other than the backbone between the Ser αC and the Pro αC is possible. Rotation primarily around the single ψ and ω bonds of pSer-Pro in a “jump rope” type of motion, where the C=O swings around, is all that is required (Figure 5).

A similar mechanism was proposed by Zhang and Noel based on the structures of cis and trans peptide inhibitors bound to Pin1, which has since been calculated using QM/MM methods.(7, 25) In order for the prolyl nitrogen lone pair to point inwards to hydrogen bond with the enzyme, the pyrrolidine ring would have to be in the less extended *cis*-pyrrolidine

conformation (referring to the ring stereochemistry, and not to be confused with the cis amide).(24) We have proposed that the stretched *trans*-pyrrolidine conformation results from strong binding of substrate phosphate and Pro–Xaa$_{i+1}$ amide carbonyl in the Pin1 active site.(24) In this work, we propose that substrate itself is likely to play a role in a strained, hydrogen-bond donation to the Pro N, similar to the proposed 5-membered ring hydrogen bond for FKBP and for peptides in solution (Figure 1).(14, 16)

CONCLUSIONS

Kinetic isotope effects (KIE) were measured to study the Pin1 catalytic mechanism of proline isomerization. We synthesized unlabeled Ac–Phe–Phe–pSer–Pro–Arg–*p*NA, and labeled Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA, and Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA substrates. For the Ser-d_3 labeled substrate, a normal 2° KIE value, k_H/k_D of 1.6 ± 0.2, indicates that the serine carbonyl hybridization is *not* changing from sp^2 to sp^3, an argument against Cys113 nucleophilic addition. This normal 2° KIE suggests a release of steric interaction between the Ser and Pro as the amide rotates. The inverse 2° KIE value of 0.86 ± 0.08 for Pro-d_7 labeled substrate shows steric crowding within the Pro ring in the transition state. A solvent KIE value, k_{H2O}/k_{D2O} of 0.92 ± 0.12, was measured, indicating that transfer of exchangeable protons is not involved. Our crystal structure of a reduced amide that resembles the twisted amide transition state suggests that a strained substrate hydrogen bond from Xaa$_{i+1}$ N–H to Pro-N is involved in the transition state. These results strongly support a twisted-amide mechanism for the Pin1 PPIase reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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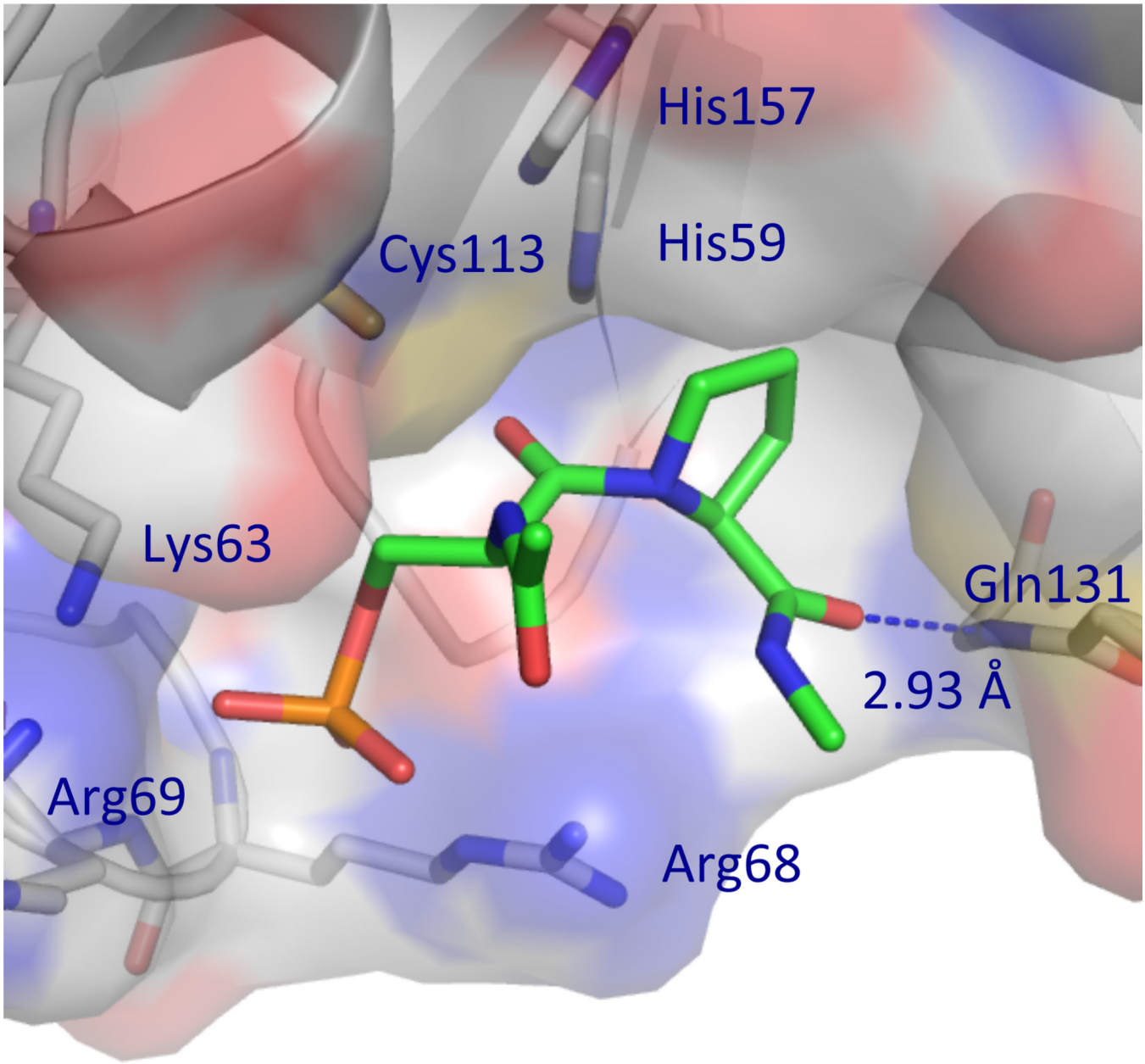

Figure 1.
The Pin1 Gln131 hydrogen bond to substrate Pro C=O positions the amide N–H for a potential 5-membered ring hydrogen bond to the pyramidalized nitrogen in the transition state. Other active site residues of Pin1 are not appropriately positioned for hydrogen bonding to the prolyl nitrogen in the transition state.(24) The crystal structure of a reduced amide-Pin1 complex was modified to show where the pSer-Pro amide carbonyl would be in a syn-exo transition state, and the tryptamine side chain was removed for clarity (3NTP). (24) (Created with MacPyMol 2006.)

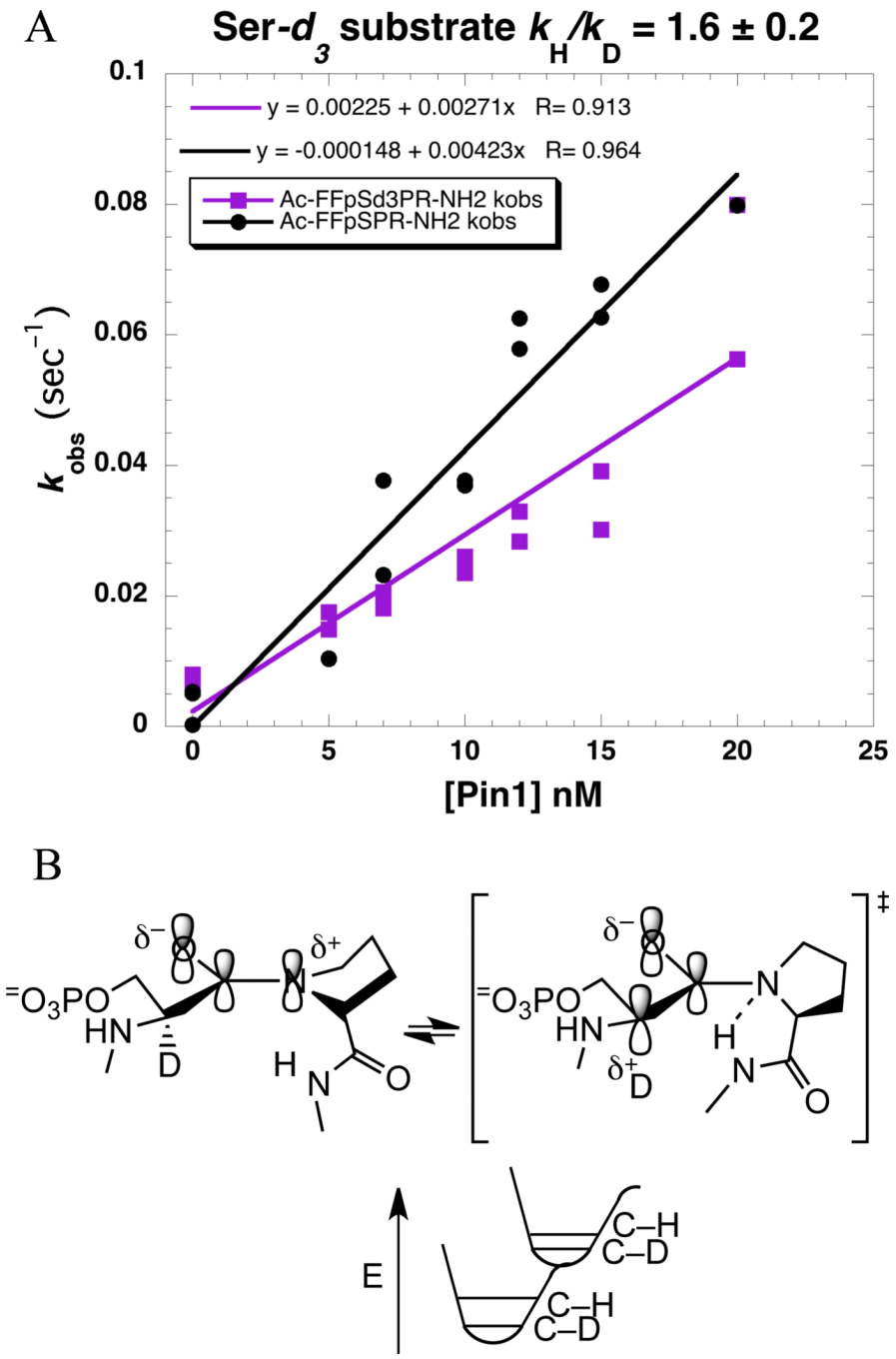

Figure 2.

(A) Experimental rate constants (k_{obs}) measured at varying Pin1 concentrations with Ac–Phe–Phe–pSer-d_3–Pro–Arg–*p*NA **2**, and unlabeled substrate **1**. (Prepared with Kaleidagraph 4.3.) (B) Hyperconjugation best explains the normal 2° KIE with the labeled Ser-d_3 substrate. The Pro-N lone pair is perpendicular to the C=O π-bond in the twisted amide transition state.

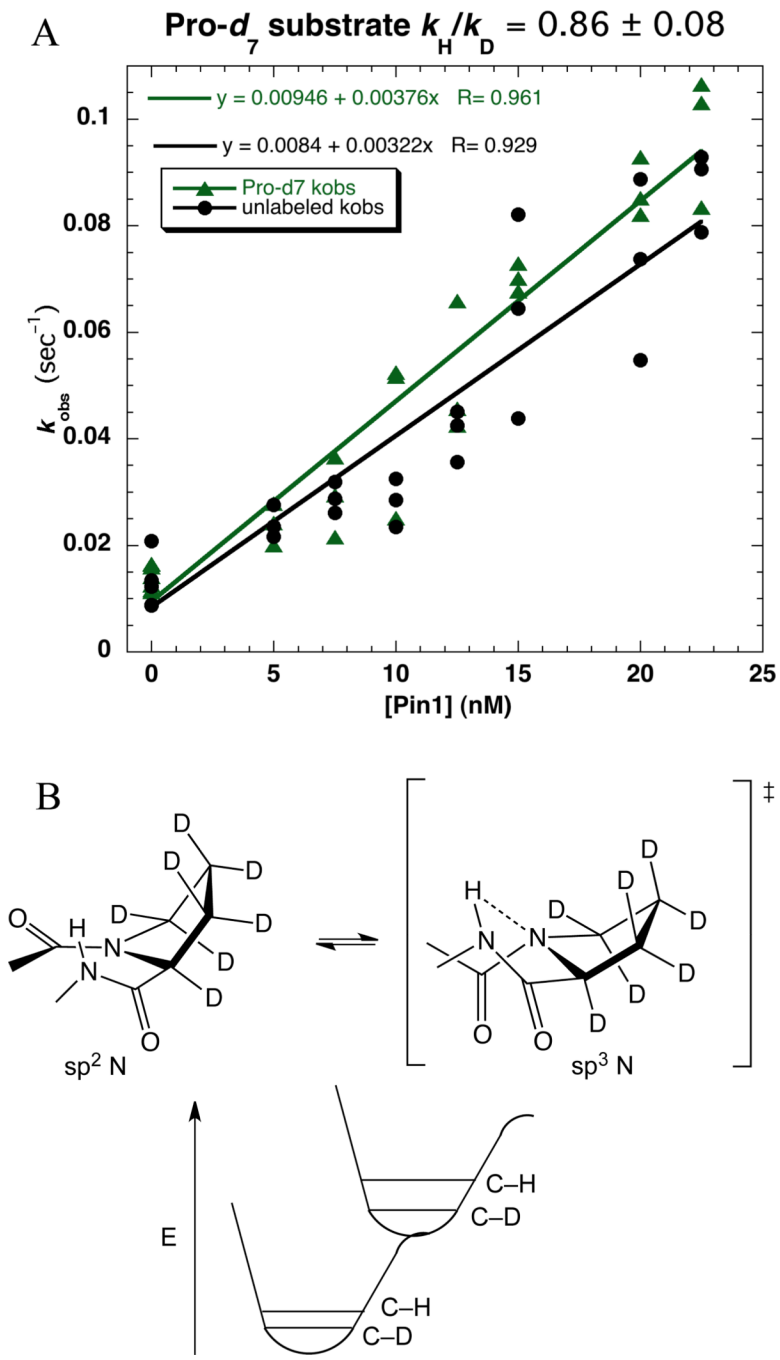

Figure 3.

(A) Experimental rate constants (k_{obs}) measured at varying Pin1 concentrations with Ac–Phe–Phe–pSer–Pro-d_7–Arg–*p*NA **3**, and unlabeled substrates. (Prepared with Kaleidagraph v. 4.3.) (B) Rehybridization of N from sp^2 to sp^3 best explains the inverse 2° KIE for the Pro-d_7 substrate.

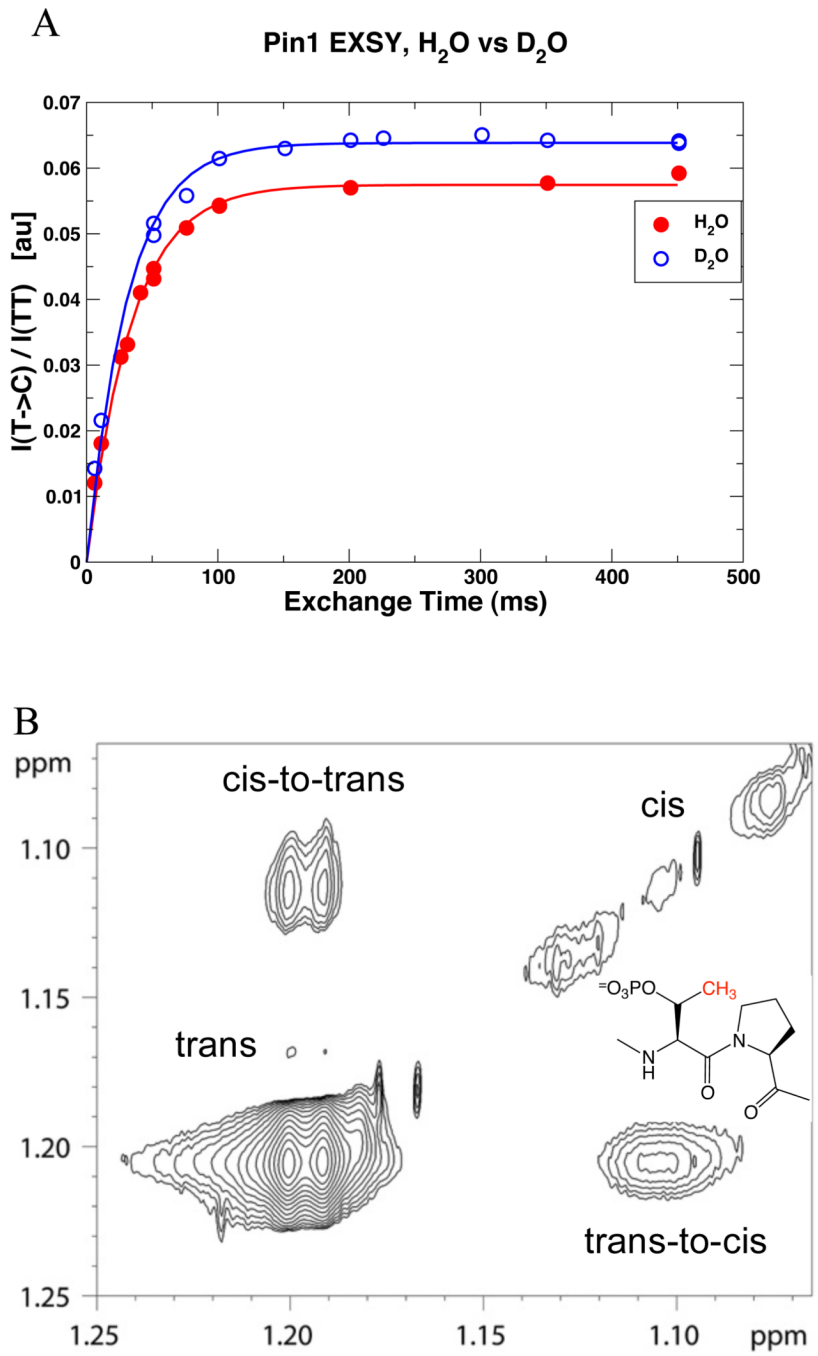

Figure 4.

(A) Experimental rate constants (k_{EX}) measured by EXSY with $^{+}H_3N$–Glu–Gln–Pro–Leu–pThr–Pro–Val–Asp–Leu–O^{-} **4** at varying exchange times in H_2O and D_2O labeled buffer giving k_{H2O}/k_{D2O} = 0.92 ± 0.12 (Prepared with Xmgrace). (B) Sample 2D EXSY D_2O (200 msec) expansion showing the cis-to-trans and trans-to-cis crosspeaks used in the measurement of k_{H2O}/k_{D2O} (Prepared with Topspin 2.1).

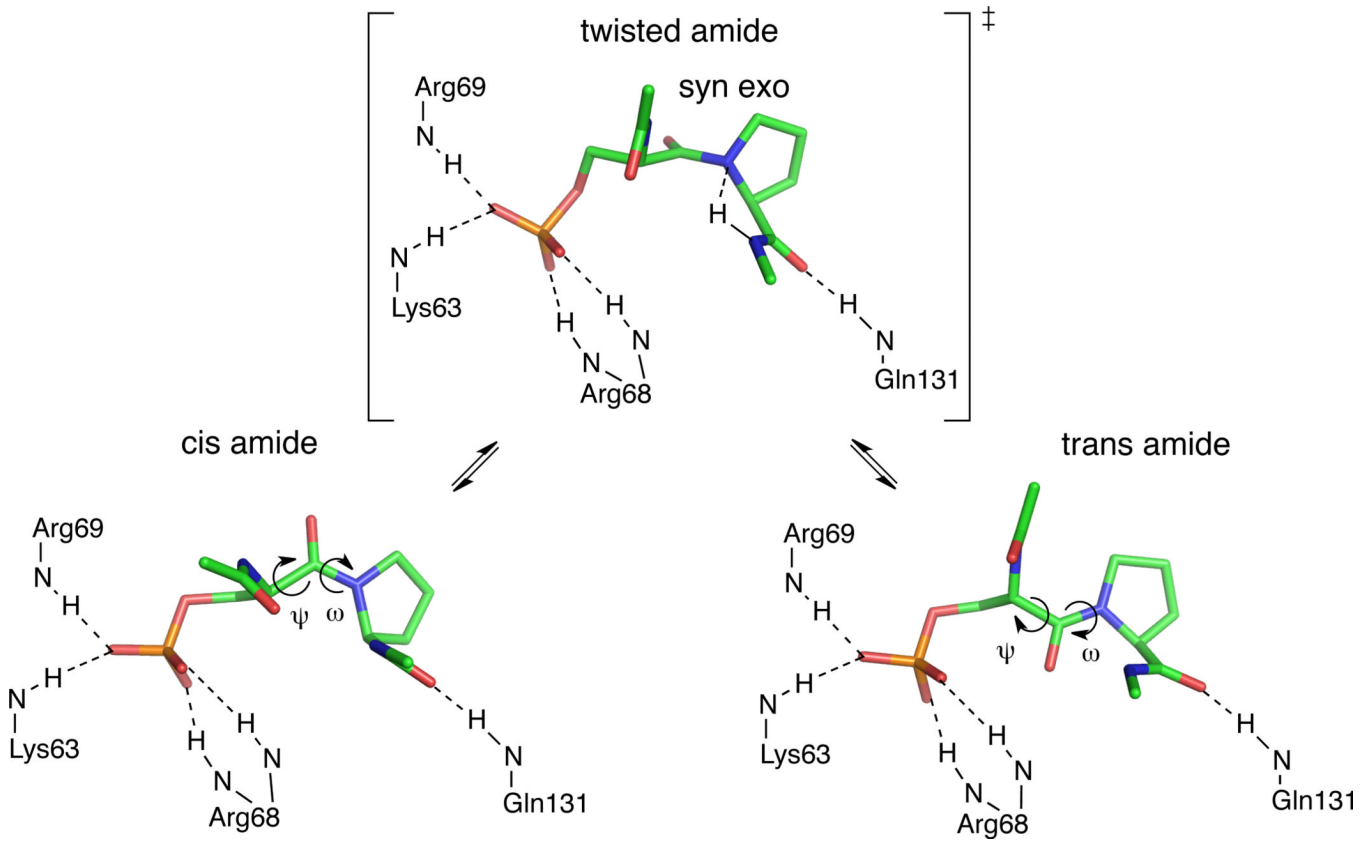

Figure 5.

Proposed mechanism of Pin1 isomerization. Crystal structures of alkene and reduced amide inhibitors of Pin1 were modified as amides to depict the bound conformations of cis (3TCZ), twisted (3NTP), and trans (3TDB) pSer–Pro amides.(24, 39) The C-terminal residues were removed, and the 3 structures were superimposed, and then separated to show the proposed twisted-amide transition state mechanism for Pin1. Hydrogen bonds between the enzyme Lys63, Arg68, Arg69 and the substrate phosphate, and between Gln131 backbone NH and substrate Pro C=O are proposed to bind the pSer–Pro amide bond in a stretched conformation to allow rotation. Little motion of groups other than the carbonyl in rotation around the ψ and ω bonds is necessary, a “jump rope” like motion of the carbonyl group. The ProN–NH_{i+1} substrate geometry in the proposed transition state is well situated for a weak 5-membered ring hydrogen bond. (Image was prepared with ChemBioDraw 12, and MacPyMol 2006.)