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

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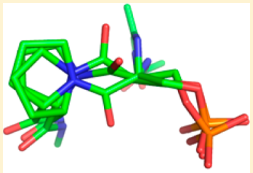
Supporting Information

ABSTRACT: The Pin1 peptidyl-prolyl isomerase 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 were collected as a function of Pin1 concentration to measure kinetic isotope effects (KIEs) on catalytic efficiency (k_{cat}/K_m). The normal secondary (2°) KIE value measured for the Ser-d_3 substrate ($k_H/k_D = 1.6 \pm 0.2$) 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 ($k_H/k_D = 0.86 \pm 0.08$) 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 ($k_{H_2O}/k_{D_2O} = 0.92 \pm 0.12$), 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.



Protein interacting with NIMA-1 (Pin1, NCBI entry 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] and (2) twisting the amide bond out of conjugation, facilitated by 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 pH between pH 5 and 9, and small, inverse solvent deuterium KIEs were measured.[10,12] For Pin1 with substrate Ala-Glu-Pro-Phe-*p*-nitroaniline, the rate was optimal between pH 6 and 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 on the basis of the bound conformation of the FK506 α-ketoamide,[12,13] as well as secondary (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 group to the prolyl N in the transition state.[14] Nonenzymatic catalysis by intramolecular hydrogen bonding was demonstrated in solution.[16] Additional evidence of N−H bond participation is that isomerization of Pro amides is much faster than that of 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 the 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 the

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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 weakened, but not total, abrogation of activity. C113A exhibited a 120-fold and C113S a 20−50-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] On the basis of 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 group hydrogen bonds to the inhibitor Pro C═O group to position the tryptamine αNH group in close proximity to the prolyl nitrogen (N−N distance of 2.63 Å) of 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, 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]

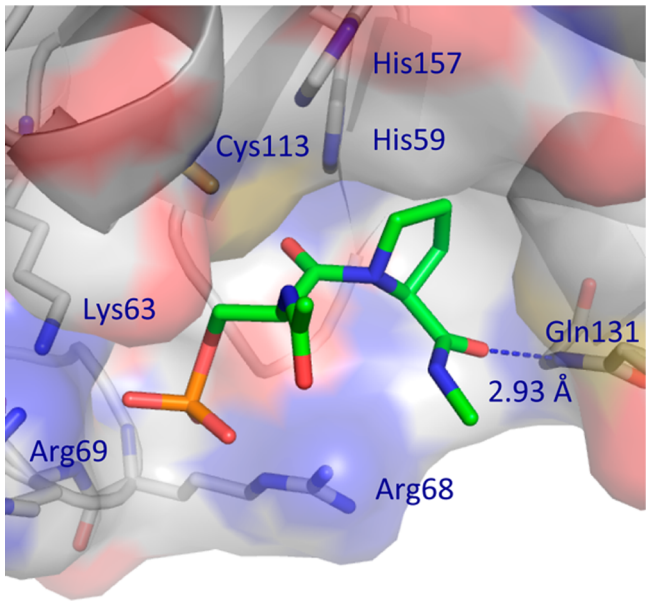

Figure 1. Pin1 Gln131 hydrogen bond to the substrate Pro C═O group that positions the amide N−H group for a potential five-membered ring hydrogen bond to the pyramidalized nitrogen in the transition state. Other active site residues of Pin1 are not appropriately positioned for hydrogen bonding to the 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 the sake of clarity (Protein Data Bank entry 3NTP)[24] (created with MacPyMol 2006).

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** were performed following the method of Bernhardt et al., with modifications as described in the Supporting Information.[26] Substrate **4** was purchased from Anaspec, Inc.[27]

Pin1 KIE 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**, respectively. Measurements for direct comparison were all taken on the same day. Concentrations of substrates **1**−**3** were determined by the UV absorbance at 390 nm (ε_{390} = 12500 M^{-1} cm^{-1}).[28] The concentrations of the cis component of substrates **1**−**3** were determined by the UV 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**, the final cis concentration was 22 μM (44% cis) and the final Ser-d_3 **2** cis concentration was 13 μM (54% cis), and (2) for unlabeled **1**, the final cis concentration was 11 μM (54% cis) and the final Pro-d_7 **3** cis concentration was 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 of 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. The substrates for experiments were as follows: (1) unlabeled 10 μL of a 5.9 mM stock and 10 μL of a 2.9 mM Ser-d_3 stock in a dry 0.48 M LiCl/TFE mixture or (2) unlabeled 7.0 μL of a 4.5 mM stock and 7.0 μL of a 5.3 mM Pro-d_7 stock in a dry 0.47 M LiCl/TFE mixture, 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 s before UV monitoring. The progress of the reaction was monitored at 390 nm for 90 s in a dry cold room to prevent the condensation of moisture on the UV cuvette during reaction monitoring.

Kinetic Analysis of Pin1 PPIase Results. Pin1 kinetics were analyzed as described by Kofron et al.[29] The absorbance versus rate data were analyzed by a nonlinear least-squares fit to an exponential equation, using TableCurve version 5.2 to measure k_{obs}.[29] Rate constants (k_{obs}) were plotted as a function of Pin1 concentration. The catalytic efficiencies, k_{cat}/K_m, were calculated from the linear fit to the plot of k_{obs} versus enzyme concentration [E] (eq 1)

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

where k_3 is the thermal background rate measured in the absence of Pin1 with each substrate.[29] The fitted value of the slope was taken to be k_{cat}/K_m, which was used to calculate kinetic isotope effect ratios. The statistical error analyses are given in the Supporting Information.

Solvent Deuterium Kinetic Isotope Effect Determined 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, deionized H_2O. The pH was adjusted to 6.60 with HCl. Cdc25 phosphopeptide EQPLpTPVTDL **4**, purchased from Anaspec Inc., was supplied with high-performance liquid chromatography and mass spectrometry analysis (>96% pure). 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 a dilute NaOH or NaOD solution. Aliquots of 55.8 μL of peptides at 12.5 mM were stored at −20 °C until they were used. Deuterated Pin1 NMR buffer was prepared with 30 mM imidazole-d_4, 30 mM NaCl, 0.03% NaN_3, and D_2O. The 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 sample 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 (bovine serum albumin standard), and then diluted to a final concentration of 50 μM in a Shigemi tube along with 2 mM pCdc25 peptide **4** and 5 mM DTT-d_{10}.

The two-dimensional (2D) ^{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 the resolution. The spectra were recorded at 295 K and 16.4T (700 MHz ^{1}H Larmor frequency). The exchange mixing times were 5, 10, 25, 30, 40, 50 (twice), 75, 100 (twice), 200, 350, and 450 ms for the protonated sample and 5, 10, 50 (twice), 75, 100, 150, 200, 225, 300, 350, and 450 ms (twice) for the deuterated sample. To estimate the net exchange rate constants, k_{EX}, we fit the ratios of the cis-to-trans exchange cross-peaks to the trans diagonal peaks as a function of mixing time t_{mix} to eq 2.[30−32]

$$\text{ratio}(t_{mix}) = \frac{(1 - e^{-k_{EX}t_{mix}})k_{TC,obs}}{k_{CT,obs} + k_{TC,obs}e^{-k_{EX}t_{mix}}} \quad (2)$$

In eq 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 Cdc25 phosphopeptide **4** without Pin1, EXSY cross-peaks were absent because the level of exchange was below the limit of detection.

RESULTS AND DISCUSSION

Unlabeled Ac-Phe-Phe-pSer-Pro-Arg-*p*NA **1** (pNA = *p*-nitroaniline) and deuterium-labeled substrates **2** and **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 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, respectively.[33,34]

Normal 2° KIE for the 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, a 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, a 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 seven 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 bond 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 βC−$(H/D)_2$ 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 substrate than the C−D substrate.[34] This steric interaction in the ground state is part of the destabilization of prolyl amides toward isomerization.[14,20]

Inverse 2° KIE for the 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 that of 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 of the 2° KIE value indicates more steric strain in the

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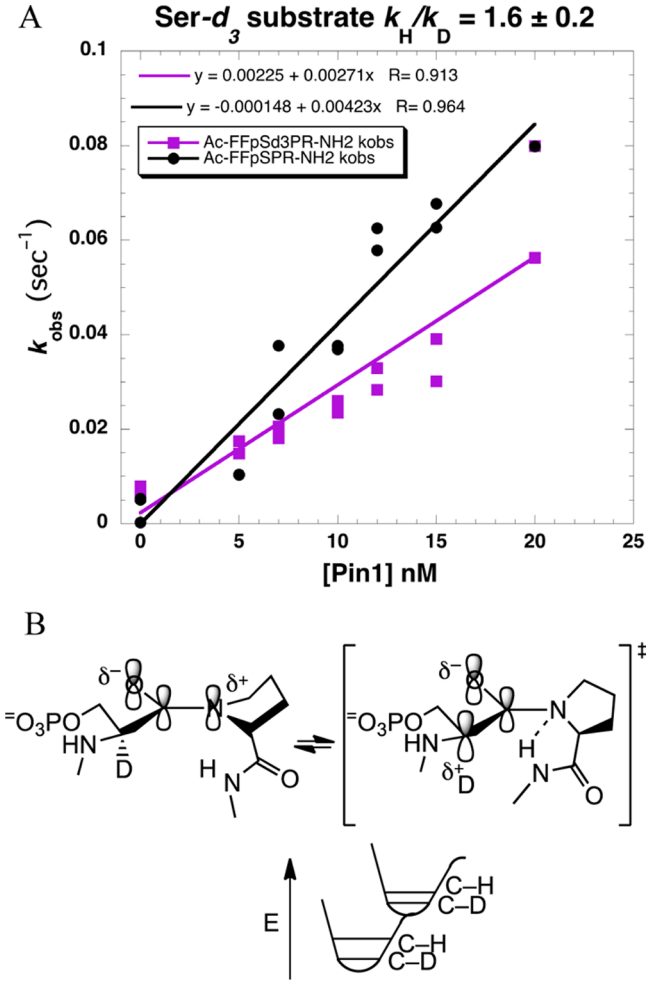

Figure 2. (A) Experimental rate constants (k_{obs}) measured at varying Pin1 concentrations with Ac-Phe-Phe-pSer-d_3-Pro-Arg-*p*NA **2** and unlabeled substrate **1** (prepared with Kaleidagraph version 4.3). (B) Hyperconjugation best explains the normal 2° KIE with the labeled Ser-d_3 substrate. The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

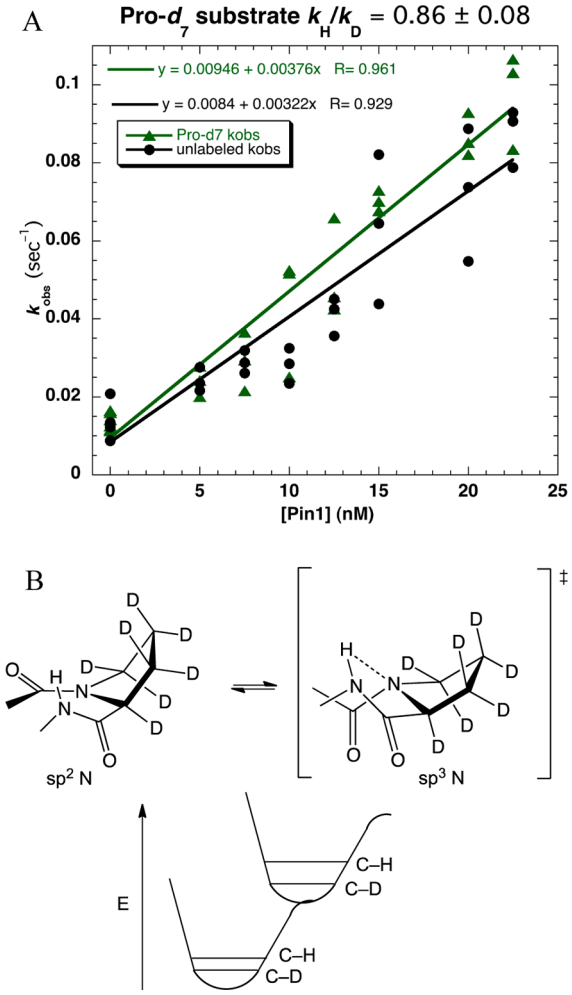

Figure 3. (A) Experimental rate constants (k_{obs}) measured at varying Pin1 concentrations with Ac-Phe-Phe-pSer-Pro-d_7-Arg-*p*NA **3** and unlabeled substrates (prepared with Kaleidagraph version 4.3). (B) Rehybridization of N from sp^2 to sp^3 best explains the inverse 2° KIE for the Pro-d_7 substrate.

transition state when the proline nitrogen rehybridizes from sp^2 to sp^3 (Figure 3B). The deuteriums the Pro-d_7 substrate are rigidly in the proximity of 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. Because trypsin, the coupling enzyme for the UV−vis-based continuous assay, has a solvent deuterium kinetic isotope effect of 1.5−4,[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, we measured the solvent deuterium isotope effect, k_{H_2O}/k_{D_2O}, 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 that in H_2O (k_{EX} = 26.2 ± 1.0 s^{-1}), giving a ratio of k_{H_2O}/k_{D_2O} of 0.92 ± 0.12 (Figure 4). The difference in the observed rates at the plateaus of the buildup 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 five-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 the NH group 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 toward 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} group of the residue after Pro (Figure 1). The NH group is, in turn, positioned by a hydrogen bond between the Pro C═O group and the NH group of Gln131 in Pin1 (Figure 2).[7,24,39] Even though the distance between the two substrate nitrogens is 2.63 Å, the N−

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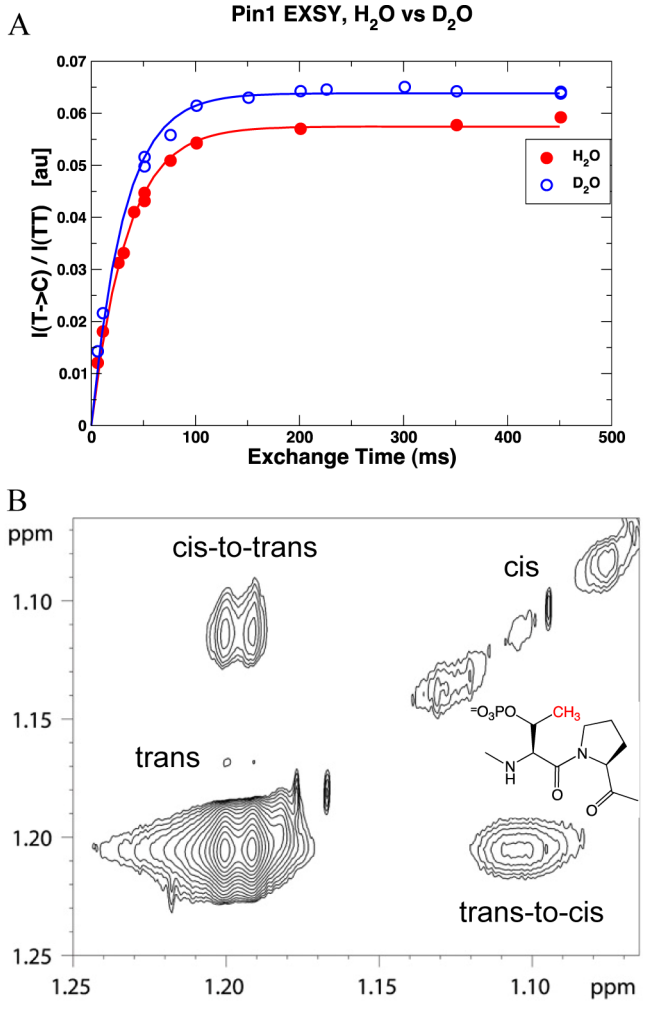

Figure 4. (A) Experimental rate constants (k_{EX}) measured by EXSY with $^{+}H_3N$-Glu-Gln-Pro-Leu-pThr-Pro-Val-Asp-Leu-O^{-} **4** at varying exchange times in H_2O and D_2O labeled buffer giving a k_{H_2O}/k_{D_2O} of 0.92 ± 0.12 (prepared with Xmgrace). (B) Sample 2D EXSY D_2O (200 ms) expansion showing the cis−trans and trans−cis cross-peaks used in the measurement of k_{H_2O}/k_{D_2O} (prepared with Topspin version 2.1).

H···N angle in such a five-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 five-membered ring, or weaker hydrogen bonding upon the 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).

Taking these results together with previously published results, we propose a mechanism for Pin1 that includes a strained hydrogen bond from the Pro-Xaa amide NH group 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, because 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 that of 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 group swings around, is all that is required (Figure 5).

A similar mechanism was proposed by Zhang and Noel on the basis of the structures of cis and trans peptide inhibitors bound to Pin1, which has since been calculated using QM/MM methods.[7,25] For the prolyl nitrogen lone pair to point inward 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, not to be confused with the cis amide).[24] We have proposed that the stretched *trans*-pyrrolidine conformation results from strong binding of the substrate phosphate and Pro-Xaa$_{i+1}$ amide carbonyl in the Pin1 active site.[24] In this work, we propose that the substrate itself is likely to play a role in the donation of strained hydrogen bond

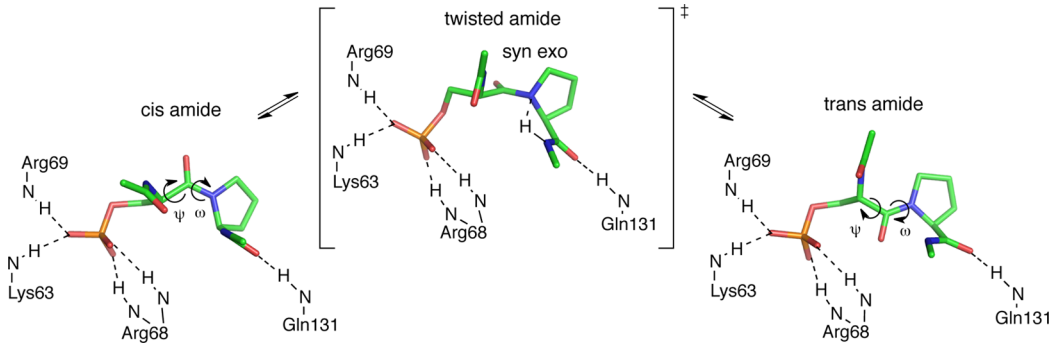

Figure 5. Proposed mechanism of Pin1 isomerization. Crystal structures of alkene and reduced amide inhibitors of Pin1 were modified as amides to depict the bound conformations of cis (PDB entry 3TCZ), twisted (PDB entry 3NTP), and trans (PDB entry 3TDB) pSer-Pro amides.[24,39] The C-terminal residues were removed, and the three structures were superimposed and then separated to show the proposed twisted amide transition state mechanism for Pin1. Hydrogen bonds between enzyme residues Lys63, Arg68, and Arg69 and the substrate phosphate and between the Gln131 backbone NH group and substrate Pro C═O group are proposed to bind the pSer−Pro amide bond in a stretched conformation to allow rotation. Little motion of groups other than that of the carbonyl in rotation around the ψ and ω angles 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 five-membered ring hydrogen bond (prepared with ChemBioDraw verison 12 and MacPyMol 2006).

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to the Pro N, similar to the proposed five-membered ring hydrogen bond for FKBP and for peptides in solution (Figure 1).[14,16]

CONCLUSIONS

KIEs 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 the Pro-d_7-labeled substrate shows steric crowding within the Pro ring in the transition state. A solvent KIE value, k_{H_2O}/k_{D_2O}, 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 the Xaa_{i+1} N−H group to Pro N is involved in the transition state. These results strongly support a twisted amide mechanism for the Pin1 PPIase reaction.

ASSOCIATED CONTENT

Supporting Information

Schemes, experimental procedures for synthesis, and NMR and mass spectrometry 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.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Lu, K. P., Hanes, S. D., and Hunter, T. (1996) A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature 380*, 544−547.

(2) Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J. U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. *Science 278*, 1957−1960.

(3) Fischer, G., Bang, H., and Mech, C. (1984) [Determination of enzymatic catalysis for the cis-trans-isomerization of peptide binding in proline-containing peptides]. *Biomed. Biochim. Acta 43*, 1101−1111.

(4) Fischer, G. (1994) Peptidyl-Prolyl *cis/trans* Isomerases and Their Effectors. *Angew. Chem., Int. Ed. 33*, 1415−1436.

(5) Ranganathan, R., Lu, K. P., Hunter, T., and Noel, J. P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. *Cell 89*, 875−886.

(6) Schroeder, O. E., Carper, E., Wind, J. J., Poutsma, J. L., Etzkorn, F. A., and Poutsma, J. C. (2006) Theoretical and Experimental Investigation of the Energetics of Cis-Trans Proline Isomerization in Peptide Models. *J. Phys. Chem. A 110*, 6522−6530.

(7) Zhang, Y., Daum, S., Wildemann, D., Zhou, X. Z., Verdecia, M. A., Bowman, M. E., Lucke, C., Hunter, T., Lu, K. P., Fischer, G., and Noel, J. P. (2007) Structural basis for high-affinity peptide inhibition of human Pin1. *ACS Chem. Biol. 2*, 320−328.

(8) Liu, J., Albers, M. W., Chen, C.-M., Schreiber, S. L., and Walsh, C. T. (1990) Cloning, expression, and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis. *Proc. Natl. Acad. Sci. U.S.A. 87*, 2304−2308.

(9) Zydowsky, L. D., Etzkorn, F. A., Chang, H. Y., Ferguson, S. B., Stolz, L. A., Ho, S. I., and Walsh, C. T. (1992) Active site mutants of human cyclophilin A separate peptidyl-prolyl isomerase activity from cyclosporin A binding and calcineurin inhibition. *Protein Sci. 1*, 1092−1099.

(10) Harrison, R. K., and Stein, R. L. (1990) Mechanistic Studies of Peptidyl Prolyl Cis-Trans Isomerase: Evidence for Catalysis by Distortion. *Biochemistry 29*, 1684−1689.

(11) Harrison, R. K., Caldwell, C. G., Rosegay, A., Melillo, D., and Stein, R. L. (1990) Confirmation of the secondary deuterium isotope effect for the peptidyl prolyl cis-trans isomerase activity of cyclophilin by a competitive, double-label technique. *J. Am. Chem. Soc. 112*, 7063−7064.

(12) Park, S. T., Aldape, R. A., Futer, O., DeCenzo, M. T., and Livingston, D. J. (1992) PPIase Catalysis by Human FK506-binding Protein Proceeds Through a Conformational Twist Mechanism. *J. Biol. Chem. 267*, 3316−3324.

(13) Rosen, M. K., Standaert, R. F., Galat, A., Nakatsuka, M., and Schreiber, S. L. (1990) Inhibition of FKBP Rotamase Activity by Immunosuppressant FK506: Twisted Amide Surrogate. *Science 248*, 863−866.

(14) Fischer, S., Michnick, S., and Karplus, M. (1993) A Mechanism for Rotamase Catalysis by the FK506 Binding Protein (FKBP). *Biochemistry 32*, 13830−13837.

(15) Wiederrecht, G., and Etzkorn, F. (1994) The immunophilins. *Perspect. Drug Discovery Des.*, 57−84.

(16) Cox, C., and Lectka, T. (1998) Intramolecular Catalysis of Amide Isomerization: Kinetic Consequences of the 5-NH-Na Hydrogen Bond in Prolyl Peptides. *J. Am. Chem. Soc. 120*, 10660−10668.

(17) Choudhary, A., and Raines, R. T. (2011) An evaluation of peptide-bond isosteres. *ChemBioChem 12*, 1801−1807.

(18) Kallen, J., and Walkinshaw, M. D. (1992) The X-ray structure of a tetrapeptide bound to the active site of human cyclophilin A. *FEBS Lett. 300*, 286−290.

(19) Howard, B., Vajdos, F., Li, S., Sundquist, W., and Hill, C. (2003) Structural insights into the catalytic mechanism of cyclophilin A. *Nat. Struct. Biol. 10*, 475−481.

(20) Hur, S., and Bruice, T. C. (2002) The mechanism of cis-trans isomerization of prolyl peptides by cyclophilin. *J. Am. Chem. Soc. 124*, 7303−7313.

(21) Behrsin, C. D., Bailey, M. L., Bateman, K. S., Hamilton, K. S., Wahl, L. M., Brandl, C. J., Shilton, B. H., and Litchfield, D. W. (2007) Functionally important residues in the peptidyl-prolyl isomerase Pin1 revealed by unigenic evolution. *J. Mol. Biol. 365*, 1143−1162.

(22) Xu, G. G., and Etzkorn, F. A. (2010) Convergent synthesis of α-ketoamide inhibitors of Pin1. *Org. Lett. 12*, 696−699.
(23) Xu, G. G., Slebodnick, C., and Etzkorn, F. A. (2012) Cyclohexyl ketone inhibitors of Pin1 dock in a trans-diaxial cyclohexane conformation. *PLoS One 7*, e44226.
(24) Xu, G. G., Zhang, Y., Mercedes-Camacho, A. Y., and Etzkorn, F. A. (2011) A reduced-amide inhibitor of Pin1 binds in a conformation resembling a twisted-amide transition state. *Biochemistry 50*, 9545−9550.
(25) Vöhringer-Martinez, E., Duarte, F., and Toro-Labbe, A. (2012) How does Pin1 catalyze the cis-trans prolyl peptide bond isomerization? A QM/MM and mean reaction force study. *J. Phys. Chem. B 116*, 12972−12979.
(26) Bernhardt, A., Drewello, M., and Schutkowski, M. (1997) The solid-phase synthesis of side-chain-phosphorylated peptide-4-nitroanilides. *J. Pept. Res. 50*, 143−152.
(27) Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. *Nature 399*, 784−788.
(28) Wang, X. J., Xu, B., Mullins, A. B., Neiler, F. K., and Etzkorn, F. A. (2004) Conformationally locked isostere of phosphoSer-*cis*-Pro inhibits Pin1 23-fold better than phosphoSer-*trans*-Pro isostere. *J. Am. Chem. Soc. 126*, 15533−15542.
(29) Kofron, J. L., Kuzmic, P., Kishore, V., Colon-Bonilla, E., and Rich, D. H. (1991) Determination of kinetic constants for peptidyl prolyl cis-trans isomerase by an improved spectroscopy assay. *Biochemistry 30*, 6127−6134.
(30) Jeener, J., Meier, B. H., Bachmann, P., and Ernst, R. R. (1979) Investigation of exchange processes by two-dimensional NMR spectroscopy. *J. Chem. Phys. 71*, 4546−4553.
(31) Kuchel, P. W., Bulliman, B. T., and Chapman, B. E. (1988) Mutarotase equilibrium exchange kinetics studied by ^{13}C-NMR. *Biophys. Chem. 32*, 89−95.
(32) Greenwood, A. I., Rogals, M. J., De, S., Lu, K. P., Kovrigin, E. L., and Nicholson, L. K. (2011) Complete determination of the Pin1 catalytic domain thermodynamic cycle by NMR lineshape analysis. *J. Biomol. NMR 51*, 21−34.
(33) Cleland, W. W. (1979) Statistical Analysis of Enzyme Kinetic Data. *Methods Enzymol. 63*, 103−138.
(34) Maitra, U., and Chandrasekhar, J. (1997) Use of Isotopes for Studying Reaction Mechanisms. 3. Secondary Kinetic Isotope Effect. *Resonance 2*, 18−25.
(35) do Amaral, L., Bull, H. G., and Cordes, E. H. (1972) Secondary Deuterium Isotope Effects for Carbonyl Addition Reactions. *J. Am. Chem. Soc. 94*, 7579−7580.
(36) Perrin, C. L., Thoburn, J. D., and Kresge, A. J. (1992) Secondary kinetic isotope effects in carbon-nitrogen rotation of amides. *J. Am. Chem. Soc. 114*, 8800−8807.
(37) Garoutte, M. P., Bibbs, J. A., and Schowen, R. L. (2006) Solvent Isotope Effects and the Question of Quantum Tunneling in Hydrolytic Enzyme Action. *J. Nucl. Sci. Technol. 43*, 455−460.
(38) Olson, L. P., Li, Y., Houk, K. N., Kresge, A. J., and Schaad, L. J. (1995) Theoretical Analysis of Secondary Kinetic Isotope Effects in C-N Rotation of Amides. *J. Am. Chem. Soc. 117*, 2992−2997.
(39) Zhang, M., Wang, X. J., Chen, X., Bowman, M. E., Luo, Y., Noel, J. P., Ellington, A. D., Etzkorn, F. A., and Zhang, Y. (2012) Structural and Kinetic Analysis of Prolyl-isomerization/Phosphorylation Cross-Talk in the CTD Code. *ACS Chem. Biol. 7*, 1462−1470.
(40) Bender, M. L., Kezdy, F. J., and Zerner, B. (1963) Intramolecular Catalysis in the Hydrolysis of p-Nitrophenyl Salicylates. *J. Am. Chem. Soc. 85*, 3017−3024.
(41) Gandour, R. D., Pivert, M. A., Kelley, E. L., Minor, B. D., Spencer, R., Ingraham, R. H., and Spindler, S. J. (1979) Solvent isotope effects in intramolecular catalysis: Acyl transfer reactions of 4-nitrophenyl 5-nitrosalicylate in aqueous tris buffer. *Bioorg. Chem. 8*, 45−58.
(42) Pesando, J. M. (1975) Proton magnetic resonance studies of carbonic anhydrase. II. Group controlling catalytic activity. *Biochemistry 14*, 681−688.
(43) Hoffmann, R., Minkin, V. I., and Carpenter, B. K. (1997) Ockham's Razor and Chemistry. *HYLE 3*, 3−28.