Substrate-Based Design of Reversible Pin1 Inhibitors†

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ABSTRACT: Human Pin1, a peptidyl−prolyl *cis/trans* isomerase with high specificity to -Ser/Thr(PO_3H_2)-Pro- motifs, is required for cell cycle progression. In an effort to design reversible Pin1 inhibitors by using a substrate structure based approach, a panel of peptides were applied to systematically analyze the minimal structural requirements for Pin1 substrate recognition. Pin1 catalysis ($k_{cat}/K_m < 5$ mM^{-1} s^{-1}) for Ala-Pro, Ser-Pro, and Ser(PO_3H_2)-Pro was detected using direct UV−visible spectrophotometric detection of prolyl isomerization, while weak competitive inhibition of Pin1 by these dipeptides was observed ($K_i > 1$ mM). Substrates with chain lengths extending from either the P2 to P1′ or the P1 to P2′ subsite gave k_{cat}/K_m values of 100 mM^{-1} s^{-1} for Ala-Ser(PO_3H_2)-Pro and 38 mM^{-1} s^{-1} for Ser(PO_3H_2)-Pro-Arg. For both Pin1 and its yeast homologue Ess1, the optimal subsite recognition elements comprise five amino acid residues with the essential Ser(PO_3H_2) in the middle position. The resulting substrate Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-4-nitroanilide possesses a very low *cis/trans* interconversion barrier in the presence of either Pin1 or Ess1, with $k_{cat}/K_m = 9300$ mM^{-1} s^{-1} and 12000 mM^{-1} s^{-1}, respectively. The D-Ser-(PO_3H_2) residue preceding proline could serve as a substrate-deactivating determinant without compromising ground state affinity. Similarly, substitution of the amide bond preceding proline with a thioxo amide bond produces a potent inhibitor. Pin1 is reversibly inhibited by such substrate analogue inhibitors with IC_{50} values in the low micromolar range. The D-amino acid containing inhibitor also exhibits remarkable stability against phosphatase activity in cell lysate.

Human Pin1, a member of the parvulin family of peptidyl−prolyl *cis/trans* isomerases (PPIases),[1] catalyzes the *cis/trans* isomerization of the imidic peptide bond in -Ser/Thr-(PO_3H_2)-Pro- moieties of polypeptides in folded and unfolded states (*1*−*3*). The C-terminal catalytic domain displays full catalytic activity only if the dianionic form of the phosphorylated Ser/Thr side chain of the substrate is present (*3*). Unphosphorylated substrates have much lower k_{cat}/K_m values, which indicate the strong impact of the phosphorylated residue on transition state binding. Interestingly, the N-terminal group IV WW domain of Pin1 (*4*) mediates -Ser/Thr-(PO_3H_2)-Pro- ground state binding, superior in affinity to the Pin1 catalytic domain (*5*, *6*). The affinity to the WW domain could be further enhanced by multiple phosphorylations of the repeated Ser-Pro sequence in either proteins or peptides (*7*).

Pin1 homologues are essential for normal progression through the eukaryotic cell cycle (*8*), and depletion of Pin1 results in premature entry into mitosis, followed by apoptosis. A *Xenopus laevis* Pin1 variant with reduced PPIase activity exhibited a compromised cell cycle rescue function in Pin1-depleted mitotic egg extracts (*9*). This suggests that the interactions between mitotic phosphoproteins and Pin1 could downregulate the initiation of mitosis and control replication check points. Surprisingly, although Pin1 appears to be a general regulator in cellular phosphorylation and dephosphorylation events, Pin1 is not critical for readily observable functions in *Drosophila melanogaster* or mice (*10*, *11*). An explanation might be that cyclophilin 18 (Cyp18) and Pin1/Ess1 act on common protein targets that would lead to functional redundancy (*12*, *13*).

To clarify the relationship between Pin1 PPIase activity and its biological functions, a dose-dependent and specific inhibition of Pin1 activity is required. Furthermore, Pin1 seems to be a good target for anticancer drug development since depletion of Pin1 induces mitotic arrest followed by apoptosis. Pin1 has been demonstrated to be a novel regulator of the oncogenic transcriptional activator β-catenin signaling (*14*). Pin1 overexpression might contribute to the upregulation of β-catenin in tumors such as breast cancer. Hitherto, no reversible inhibitor is known for Pin1. In a first attempt, the brown walnut dye juglone (5-hydroxy-1,4-naphthochinone) has been shown to inhibit Pin1, without inhibition of the PPIases of other families. The inactivation mechanism involves partial unfolding of the active site following covalent modification of an active site cysteine (*15*). In agreement with the proposed function for Pin1, juglone induced apoptosis and blocked entry into mitosis by interphase arrest in cancer cells (*16*). Protective effects against

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[1] Abbreviations: Ac, acetyl; δ, chemical shift; CE, capillary electrophoresis; DIPEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; ESI-MS, electrospray mass spectroscopy; FKBP, FK506 binding protein; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; ISP, isomer-specific proteolysis; NH-Np, 4-nitroanilide; PPIase, peptidyl−prolyl *cis/trans* isomerase; Suc, succinyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

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intestinal neoplasia have also been observed for juglone by dietary exposure in rats (*17*). However, the high chemical reactivity of juglone toward other proteins prevents reliable conclusions being made from the inhibition data about the involvement in cellular Pin1 action (*18*).

The standard spectrophotometric assay for PPIases (*19*) allows the investigation of only one direction of the reversible prolyl isomerization and is restricted to reaction components stable toward the appropriate protease. These disadvantages have been circumvented by newly developed protease-free spectrophotometric and fluorometric assays (*20*−*22*). However, all of these assays make use of substrates containing artificial moieties that prevent the in vitro characterization of naturally occurring PPIase substrates. In this study, we have identified the minimal subsite requirements for Pin1 and Ess1 catalysis by using a novel assay that could detect prolyl bond isomerization of small natural substrates such as Ala-Pro.[2] On the basis of these results, we designed substrates with minimal chain length and high transition state affinity. The conversion of substrates into inhibitors was accomplished by the incorporation of the nonnatural amino acid building block D-Ser(PO_3H_2) or peptide bond mimic -Ψ-[CS-N]-Pro- into the appropriate position.[4,5] Exhibiting high Pin1 affinity, the natural peptide mimics did not inhibit PPIases of other families and may serve to facilitate studies on the physiological role of the enzyme activity of Pin1.

EXPERIMENTAL PROCEDURES

Materials. The Pin1 vector was from K. P. Lu (*23*). The purified PPIases Pin1 and Ess1 were kindly provided by J.-U. Rahfeld. The proteases α-chymotrypsin and trypsin were products from Merck (Darmstadt, Germany). The subtilisin-like protease nagarse was purchased from Sigma Chemical Co. (St. Louis, MO).

The peptide derivatives Suc-Ala-Ala-Pro-Phe-NH-Np, Suc-Ala-Pro-Phe-NH-Np, Suc-Ala-Glu-Pro-Phe-NH-Np, Ala-Pro, Ser-Pro, Ala-Pro-Ala, Ala-Ala-Pro, and Ala-Ala-Pro-Ala were purchased from Bachem (Heidelberg, Germany). The syntheses of the phosphorylated peptides Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np and Ser(PO_3H_2)-Pro-Arg-NH-Np were carried out according to Schutkowski and co-workers (*3*). Ac-Ala-Ala-Pro-Ala-Lys-NH_2 was synthesized as described previously (*24*). The peptide Ser(PO_3H_2)-Pro-Arg was obtained through tryptic digestion of Ser(PO_3H_2)-Pro-Arg-NH-Np. The phosphopeptides Ser(PO_3H_2)-Pro, Ala-Ser(PO_3H_2)-Pro, and Ala-Ser(PO_3H_2)-Pro-Arg were synthesized by solid-phase peptide synthesis using chlorotrityl chloride resin, TBTU, HOBt (Alexis Biochemicals), Fmoc-Ser(PO(OBzl)-OH)-OH (Calbiochem Novabiochem AG) as phosphoamino acid building block, and dimethylformamide as a reaction solvent. D-Ser(PO_3H_2)-Pro and Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np were synthesized by solid-phase peptide synthesis using Fmoc-D-Ser(P(O)($OBzl)_2$) as the phosphoamino acid building block and PyBOP as the coupling reagent. After detachment of the peptides from the resin and side-chain deprotection with TFA, these phosphopeptides were purified by RP8 HPLC and lyophilized. Ser-Ψ[CS-N]-Pro was synthesized from Boc-Ser-Pro-OBu-*t* through thioxylation with P_4S_{10} in THF and deprotected in TFA. The resulting crude product was purified with RP8 HPLC and lyophilized. Fmoc-NHPhe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np was phosphorylated by (*i*-Pr_2N)P($OBzl)_2$/tetrazole in THF at room temperature and oxidated by *tert*-butyl hydroperoxide at −15 °C. After deprotection with the $ZnCl_2/Et_2O$ complex under argon and then with piperidine, it was purified by RP8 HPLC and lyophilized. Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np was phosphorylated by (*i*-Pr_2N)P($OAll)_2$/tetrazole in THF and oxidated by *tert*-butyl hydroperoxide at room temperature. Deprotection of allyl ester was performed in THF with tetrakis(triphenylphosphine)palladium/butylamine/formic acid at 50 °C for 4 h. After separation by RP8 HPLC, the peptide was lyophilized. Peptide identity was confirmed by ^1H, ^{13}C, and ^{31}P NMR and/or by ESI-MS. The purity of peptides was checked by CE and/or RP18 HPLC.

PPIase Assay. The PPIase activity was monitored at 10 °C with a protease-coupled assay on a Hewlett-Packard 8452a diode array spectrophotometer as described previously (*19*). Pin1 and Ess1 activities were measured with the phosphorylated peptides Ser(PO_3H_2)-Pro-Arg-NH-Np and Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np and with the unphosphorylated peptides Suc-Ala-Pro-Phe-NH-Np and Suc-Ala-Ala-Pro-Phe-NH-Np as substrates. Trypsin or α-chymotrypsin was used as the auxiliary protease. The peptides were dissolved in DMSO (10 mg/mL), with the exception of the dipeptides, which were dissolved in 20 mM Tris, pH 7.8. Inhibition data for dipeptides were analyzed according to the competitive model as already published for cyclophilin 18 (*25*). The residual enzymatic activity was plotted versus the inhibitor concentration and used for calculating the inhibitory constant (*26*). The IC_{50} values of the competitive substrates Ser(PO_3H_2)-Pro-Arg and Ser(PO_3H_2)-Pro-Arg-NH-Np were determined for Pin1 at 10 °C in 20 mM Tris, pH 7.8, using the substrate Suc-Ala-Glu-Pro-Phe-NH-Np as the reporter substrate and nagarse as the auxiliary protease. The inhibition constant of the substrate-derived inhibitors for Pin1 was measured at 7 °C in 35 mM HEPES, pH 7.8, using Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np as the substrate.

The determination of the kinetic constants of the *cis/trans* isomerization within a protease-free system was performed using fluorescence measurements according to Garcia-Echeverria et al. (*21*). These measurements were performed on a Hitachi F-3010 fluorescence spectrophotometer at 10 °C with Abz-Ala-Glu-Pro-Phe-NH-Np as the substrate. The substrate (10 mM) was dissolved in anhydrous trifluoroethanol/0.5 M LiCl. The measurement of the *cis/trans* isomerization was initiated by addition of the substrate solution to a mixture of 35 mM HEPES, pH 7.8 (final concentration 20 μM), 22 nM Pin1, and inhibitor of desired concentrations. The excitation wavelength was 320 nm with a spectral bandwidth of 3 nm. Emission was detected at 416 nm with a spectral bandwidth of 10 nm. Data analysis was performed by single-exponential nonlinear regression using the SigmaPlot Scientific Graphing System Version 2.00 (Jandel Corp.). Determination of the IC_{50} values was performed as described previously (*26*).

Stability of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np in Cell Lysate. The Pin1 inhibitor Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np was incubated with cell lysate (whole cell

[2] The term prolyl bond used throughout the paper refers to the peptide bond preceding proline in a peptide sequence, while prolyl isomerization refers to the *cis/trans* isomerization of the peptide bond preceding proline.

Table 1: Influence of pH and Solvent on the Thermodynamic Equilibrium of Prolyl Bonds and the Resulting Relative Changes of the UV Signal

Peptide	Method	*cis* content [%][g]			Δ Signal (Change after jump) [%]
		pH 2.1	pH 7.8	0.5M LiCl/TFE	
Ala-**Pro**	pH-jump [a,d]	10	39		+7
Ala-Ala-**Pro**	pH-jump [a,d]	8	14		+2
	solvent-jump [a,e]			37	-6
Ala-**Pro** -Ala	pH-jump [b,d]	10	11		0
	solvent-jump [b,e]			17	-0.8
Ala-Ala-**Pro** -Ala	pH-jump [a,d]	5	10		0
	solvent-jump [a,e]			37	-4
Ac-Ala-Ala-**Pro** -Ala-Lys-NH_2	solvent-jump [a,e]		8	23	-2
Ser-**Pro**	pH-jump [a,d]	20	40		+5
Ser(PO_3H_2)-**Pro**	pH-jump [a,d]	12	18		+8
Ser(PO_3H_2)-**Pro** -Arg	pH-jump [c,d]	6	17		+2
Ala- Ser(PO_3H_2)-**Pro**	solvent-jump [b,f]		23	n.d.	-0.5
Ala- Ser(PO_3H_2)-**Pro** -Arg	solvent-jump [b,e]		16	n.d.	+0.5

[a] Measured at 220 nm. [b] Measured at 210 nm. [c] Measured at 215 nm. [d] pH jump between the cationic form (50 mM H_3PO_4, pH 2.1) and zwitterionic form (20 mM Tris, pH 7.8). [e] Solvent jump from a 25−50 mM solution in 0.5 LiCl/TFE to 20 mM Tris, pH 7.8. [f] Solvent jump from a 50 mM solution in ethanol to 20 mM Tris, pH 7.8. [g] Determined by ^{1}H NMR spectroscopy. nd, not determined.

lysate of HeLa cellls) for 15 min, 1 h, 2 h, and 3 h and analyzed with capillary electrophoresis (CE), using Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np as a reference. CE using UV detection was performed on P/ACE MDQ (Beckman Coulter, Palo Alto, CA). Fused-silica capillaries (Polymicro Technologies) of 60 cm length (50 cm to the detection window) and 50 μm i.d. were used. The operation voltage was 30 kV. Hydrodynamic injection at a pressure of 0.5 psi (3.45 kPa) for 10 s was applied to the incubated sample. The capillary thermostating system was kept at 25 °C. Separation buffer was 50 mM phosphate buffer, pH 8.0. Detection was performed at 200, 280, and 320 nm.

Direct UV Detection of the Peptidyl−Prolyl Cis → Trans Isomerization. A Zeiss M500 double beam spectrophotometer (Jena) with continuous stirring and a thermostated cuvette holder was used for the UV measurements. The peptides were dissolved in 50 mM HCl or phosphoric acid with a final pH of 2.1. The 50−100-fold dilution of these stock solutions into 20 mM Tris, pH 7.8, gave a final peptide concentration of 0.3−0.7 mM and led to an increase of the content of *cis* isomers (Table 1). The *cis*/*trans* equilibration at pH 7.8 was followed at 210 or 215 or 220 nm dependent on the largest absorbance change during the interconversion. The final absorbance was adjusted to approximately 1.5 to guarantee the reliable detection of the relatively small signal change of 1−7% in the overall absorbance. The Δ signal was defined as the percentage of an UV signal change responding to a pH (or solvent) jump as a factor for the assay quality.

The apparent first-order rate constants k_{obs} were computed by nonlinear regression of the progress curves. The determined k_{obs} values are the sum of the separate rate constants for the *cis* → *trans* and the *trans* → *cis* isomerization (eq 1). The microscopic rate constants $k_{c\to t}$ and $k_{t\to c}$ were calculated on the basis of the known equilibrium constant K_{eq} (eq 2). For comparisons with the data from protease-coupled tests and the known activation parameters from the literature, only the rate constants of the *cis* → *trans* isomerization were used.

$$k_{obs} = k_{c\to t} + k_{t\to c} \quad (1)$$

$$K_{eq} = [trans]/[cis] = k_{c\to t}/k_{t\to c} \quad (2)$$

The *cis* → *trans* isomerizations ($k_{c\to t}$) of di- and tripeptides were measured at different temperatures, and the data were fitted to the Arrhenius plot to determine the thermodynamic parameters.

The substrate properties of the peptides were investigated by addition of 50−150 nM PPIase (Pin1, Ess1). The first-order rate constants for the PPIase-catalyzed *cis* → *trans* isomerization ($k^e_{c\to t}$) were obtained by subtraction of the uncatalyzed isomerization (k^u) from the observed first-order rate constant (eq 3) and by combination with eq 2.

$$k_{obs} = k^u + k^e = (k^u_{c\to t} + k^u_{t\to c}) + (k^e_{c\to t} + k^e_{t\to c}) \quad (3)$$

Alternatively, using solvent jump, peptides Ala-Ala-Pro, Ala-Pro-Ala, Ala-Ala-Pro-Ala, Ac-Ala-Ala-Pro-Ala-Lys-

Table 2: Calculated Molar Extinction Coefficients for the *Cis* and *Trans* Isomers of Ala-Pro in the Cationic Form (pH 2.1) and in the Zwitterionic Form (pH 7.8)[a]

	cation[b]		zwitterion[c]	
λ (nm)	ϵ_{cis} ($M^{-1}\,cm^{-1}$)	ϵ_{trans} ($M^{-1}\,cm^{-1}$)	ϵ_{cis} ($M^{-1}\,cm^{-1}$)	ϵ_{trans} ($M^{-1}\,cm^{-1}$)
200	8208	7428	7784	7395
205	7800	6864	7922	7377
210	6272	5300	7180	6449
215	4344	3440	5725	4910
220	2672	1908	3916	3132
225	1404	880	2210	1635
230	648	308	1012	677

[a] Calculation of ϵ_{cis} and ϵ_{trans} as described in Experimental Procedures. [b] Evaluated from recorded time-resolved UV spectra at 30 and 1200 s after starting the 100-fold dilution of 20 mM Ala-Pro in sodium phosphate, pH 7.8, into 20 mM sodium phosphate, pH 2.1. [c] Evaluated from recorded time-resolved UV spectra at 30 and 1200 s after starting the 100-fold dilution of 20 mM Ala-Pro in sodium phosphate, pH 2.1, into 20 mM sodium phosphate, pH 7.8.

NH_2, Ala-Ser(PO_3H_2)-Pro, and Ala-Ser(PO_3H_2)-Pro-Arg were tested as PPIase substrates in a similar way. The increased *cis* contents of these oligopeptides in 0.5 M LiCl/anhydrous TFE [in the case of Ala-Ser(PO_3H_2)-Pro in ethanol] declined during reequilibration after 50-fold dilution into the buffered medium (20 mM Tris, pH 7.8) and led to a signal change of 0.5−6% at 210−220 nm (Table 1).

Calculation of the Molar Absorption Coefficients of the Cis and Trans Isomers of Ala-Pro at Different Protonation States. To estimate the molar absorption coefficients of the *cis* and *trans* isomers of Ala-Pro at different ionization states, time-resolved UV spectra were recorded. Using the known amounts of the *cis* and *trans* isomers (measured by ^{1}H NMR) and the Ala-Pro concentration, the molar absorption coefficients of 100% *cis* and 100% *trans* were calculated by combining the Lambert−Beer law and the equation:

$$A_i = \epsilon_{100\%cis}[\text{cis}]_i + \epsilon_{100\%trans}[\text{trans}]_i \qquad (4)$$

where A_i is the absorbance of Ala-Pro at ionization state *i* and the light path is 1 cm.

The determined parameters are shown in Table 2. The molar absorption coefficients at 220 nm were confirmed by recalculation from time course measurements after pH jumps in both directions.

RESULTS

The slow reequilibration of the prolyl bonds to a different *cis/trans* ratio of proline-containing oligopeptide following a rapid-jump protocol provides means for the direct monitoring of *cis/trans* isomerization. UV spectra of Ala-Pro after a pH jump revealed a difference (Tables 1 and 2) between the *cis* and the *trans* isomers in the wavelength range between 200 and 240 nm (Figure 1, insets) with a higher absorbance for the cis isomer. The time-dependent change of the UV signal following the pH jump can be expressed in terms of strict first-order kinetics (Figure 1). The calculated first-order rate constants represent the sum of the rate constants for the spontaneous *cis* → *trans* and *trans* → *cis* isomerizations. The inverse curve shape, as shown in the time course of the signal following the jump from pH 2.1 to pH 7.8 (Figure 1A) or the jump from pH 7.8 to pH 2.1 (Figure 1B), indicates the

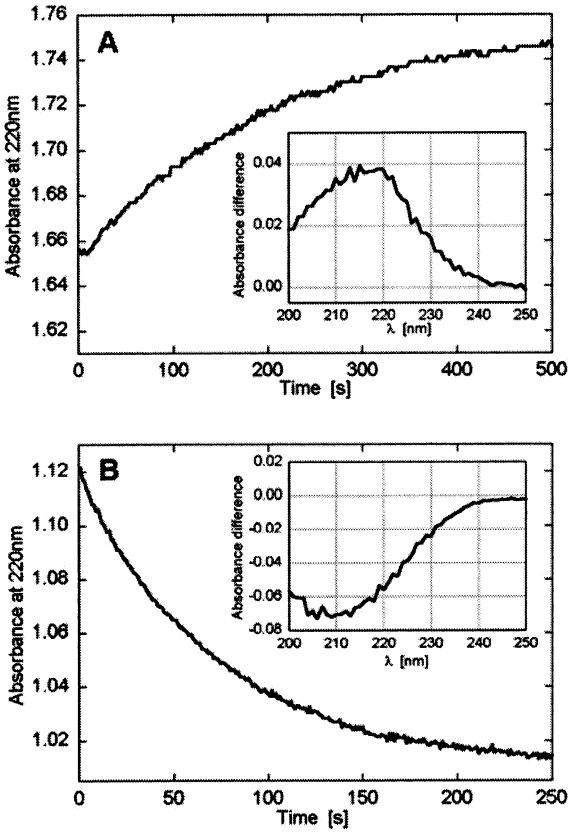

FIGURE 1: Time course of the reversible first-order prolyl isomerization of Ala-Pro following a pH jump at 25 °C (A, B) and difference spectra given in the inserts. (A) pH jump from a 25 mM stock solution in sodium phosphate, pH 2.1, to 20 mM sodium phosphate, pH 7.8, monitored at 220 nm. The final peptide concentration was 0.5 mM, and the final *cis* content was 39%. A first-order rate constant k_{obs} of 5.0×10^{-3} s^{-1} was calculated, which is the sum of the rate constants $k_{c\rightarrow t} = 3.1 \times 10^{-3}$ s^{-1} and $k_{t\rightarrow c} = 1.9 \times 10^{-3}$ s^{-1}. (B) pH jump from a 50 mM stock solution in sodium phosphate, pH 7.8, to 20 mM sodium phosphate, pH 2.1, monitored at 220 nm. The final peptide concentration was 0.5 mM, and the final *cis* content was 10%. A first-order rate constant k_{obs} of 1.4×10^{-2} s^{-1} was calculated, which is the sum of the rate constants $k_{c\rightarrow t} = 1.2 \times 10^{-2}$ s^{-1} and $k_{t\rightarrow c} = 1.4 \times 10^{-3}$ s^{-1}. The insets in (A) and (B) represent the difference spectra of 0.2 mM Ala-Pro calculated from spectra at 30 and 1200 s after starting a jump from pH 2.1 to pH 7.8 and from pH 7.8 to pH 2.1, respectively. A dilution jump without change of pH (measured at pH 2.1 and pH 7.8) resulted in baseline spectra.

reversibility of the reaction underlying pH jumps. Activation parameters, as shown in Table 3, were determined by a combination of reversible isomerization constants measured at different temperatures with the direct assay and the NMR-derived equilibrium constants. The rate constants (Figure 2) exhibit linear Arrhenius plots with Arrhenius activation energies E_A in the range of 79−93 kJ/mol for both phosphorylated and unphosphorylated dipeptides and tripeptides, depending on both the final conditions for reequilibration and the peptide structure (data not shown). They are within the typical range for prolyl bond isomerization of oligopeptides. However, a strong pH influence on the *cis* content, which is required for pH-jump experiments, cannot be observed for peptides lacking the C-terminal proline. Alternatively, the isomerization assay can also be performed by solvent-jump experiments that make use of dry mixtures of TFE/LiCl as solvent. The time courses of solvent jump and pH jump both ended under identical buffer conditions and

Table 3: Activation Parameters of the *Cis* → *Trans* Isomerization of Di- and Tripeptides after the pH Jump as Calculated According to the Arrhenius Equation ($k_{c\rightarrow t} = Ae^{-E_A/RT}$)[a]

peptide[b]	pH jump	final *cis* content (%)[e]	E_A (kJ/mol)	ln *A*
Ala-Pro[c]	2.1 → 7.8	39	92.1	31.4
Ala-Pro[c]	7.8 → 2.1	10	79.1	26.0
Ser-Pro[c]	2.1 → 7.8	40	85.5	30.2
Ser(PO_3H_2)-Pro[c]	2.1 → 7.8	18	93.2	32.6
Ser(PO_3H_2)-Pro-Arg[d]	2.1 → 7.8	17	83.1	30.0

[a] Determined as described in the legend of Figure 2. [b] The final concentrations were 0.5 mM Ala-Pro, 0.4 mM Ser-Pro, 0.7 mM Ser(PO_3H_2)-Pro, and 0.36 mM Ser(PO_3H_2)-Pro-Arg in 20 mM sodium phosphate. [c] Monitored at 220 nm. [d] Monitored at 215 nm. [e] Measured by ^{1}H NMR spectroscopy.

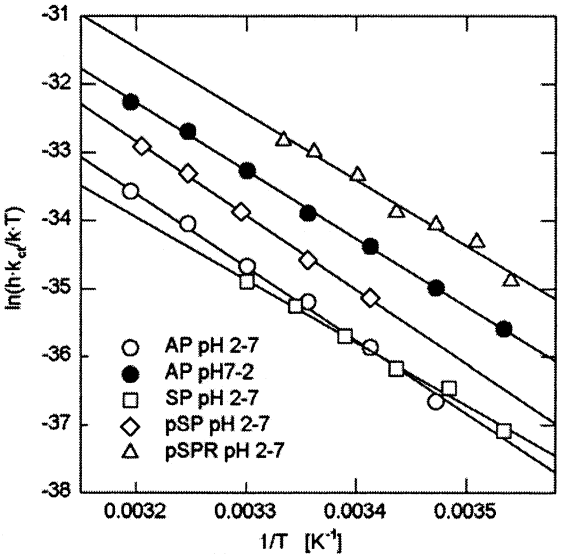

FIGURE 2: Arrhenius diagram for temperature-dependent *cis* → *trans* isomerization of Ala-Pro (○), Ser-Pro (□), Ser(PO_3H_2)-Pro (◇), and Ser(PO_3H_2)-Pro-Arg (△) after a jump from pH 2.1 to pH 7.8 and of Ala-Pro (●) after a jump from pH 7.8 to pH 2.1. Rate constants were determined by UV measurements at 220 nm [for Ser(PO_3H_2)-Pro-Arg at 215 nm] at temperatures between 10 and 45 °C. The final peptide concentrations were 0.5 mM for Ala-Pro, 0.4 mM for Ser-Pro, 0.7 mM for Ser(PO_3H_2)-Pro, and 0.36 mM for Ser(PO_3H_2)-Pro-Arg in 20 mM sodium phosphate of desired pH.

gave similar first-order rate constants. Slight differences in solvent composition (2% TFE and 10 μM LiCl in the solvent-jump experiment) do not contribute significantly to the *cis* content that is approximately 14% in these two systems. Both jump experiments also indicate that the observed absorbance change is governed by the reversible *cis/trans* isomerization (Figure 3).

The sensitivity of the assay for a given peptide, however, depends on both the difference of the molar extinction coefficients of the isomers and the difference in the *cis* content of the peptide before and after the jump. Independent measurements by ^{1}H NMR spectroscopy were used to determine the *cis/trans* isomer ratio under the respective conditions (Table 1). The percentage of UV signal change responding to a pH (or solvent) jump (Δ signal) as a factor for the assay quality (Table 1) was also determined. A high value of Δ signal represents a large time-dependent increase of the absorbance at the given wavelength and corresponds to a favorable signal-to-noise ratio.

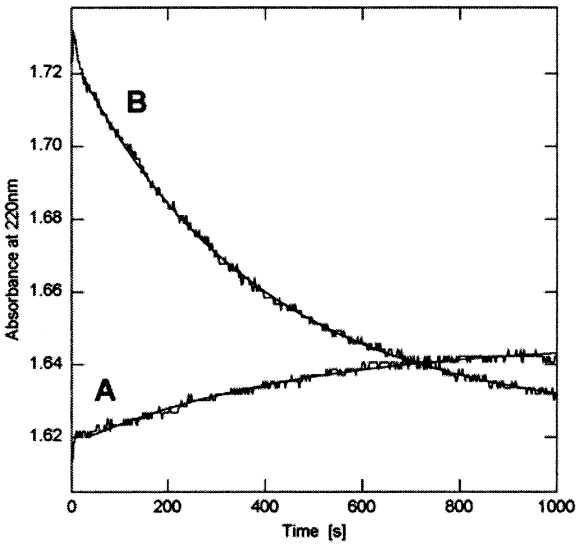

FIGURE 3: Reequilibration of Ala-Ala-Pro after pH or solvent jump at 20 °C. The absorbance changes at 220 nm were followed after 50-fold dilution of a 25 mM stock solution of Ala-Ala-Pro, pH 2.1 (A), or of a 25 mM stock solution in 0.5 M LiCl/TFE (B) into 20 mM Tris, pH 7.8 (14% *cis* content). The solid lines represent the fit of the experimental data according to a first-order rate equation. The k_{obs} values were calculated with values of 2.5×10^{-3} s^{-1} and 2.6×10^{-3} s^{-1}, respectively.

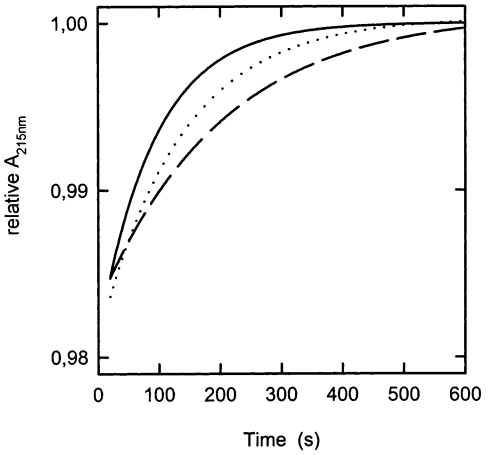

FIGURE 4: Catalysis of the *cis/trans* isomerization of Ser(PO_3H_2)-Pro-Arg by Pin1 and Ess1. The absorbance was followed at 220 nm in 20 mM Tris, pH 7.8 at 10 °C. The final substrate concentration was 0.36 mM after a 140-fold dilution of 50 mM stock solution at pH 2.1. The first-order rate constants were determined in the presence of 124 nM Pin1 (solid line) or 104 nM Ess1 (dotted line) or without addition of enzyme (dashed line). The k_{cat}/K_m is 38 mM^{-1} s^{-1} for Pin1 and 7.4 mM^{-1} s^{-1} for Ess1.

Increased first-order rate constants of *cis/trans* interconversion in the presence of Pin1 reflect the effect of enzyme catalysis (Figure 4). Although the principal structural requirements for a substrate were already present in the dipeptide Ser(PO_3H_2)-Pro, Pin1 catalysis was not found. Pin1 and Ess1 behave similarly in their catalytic action. They require a tripeptide as the minimal substrate structure. As shown in Table 4, the specificity constants k_{cat}/K_m reflect the impact of the peptide length on the catalysis by Pin1 and Ess1. The addition of only one amino acid residue to Ser(PO_3H_2)-Pro switches the competitive inhibitor to a substrate with k_{cat}/K_m values of 100 mM^{-1} s^{-1} for Ala-Ser-(PO_3H_2)-Pro and 38 mM^{-1} s^{-1} for Ser(PO_3H_2)-Pro-Arg (Table 4). Further elongation resulted in a very efficient

Table 4: Influence of the Human Parvulin Pin1 and the Yeast Parvulin Ess1 on the Kinetics of the *Cis* → *Trans* Isomerization of Various Peptides[a]

Peptide		Pin1[g]	Ess1[g]
	$k_{c\rightarrow t}$ at 10°C (10^3 s^{-1})	k_{cat}/K_M ($mM^{-1}s^{-1}$)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
Ala-**Pro**[b]	0.42	no catalysis (K_i=19 mM)	no catalysis
Ala-Ala-**Pro**[b,c]	0.42	no catalysis	no catalysis
Ala-**Pro** -Ala[c]	4.66	no catalysis	no catalysis
Ala-Ala-**Pro** -Ala[c]	7.70	9	8
Ac-Ala-Ala-**Pro** -Ala-Lys-NH_2[c]	4.57	29	12
Suc-Ala-**Pro** -Phe-NH-Np[e]	5.81	21	6
Suc-Ala-Ala-**Pro** -Phe-NH-Np[e]	7.20	21	7
Ser-**Pro**[b]	0.46	no catalysis (K_i=27 mM)	no catalysis
Ser(PO_3H_2)-**Pro**[b]	0.86	no catalysis (K_i=1 mM)	no catalysis
Ser(PO_3H_2)-**Pro** -Arg[b]	4.26	38[h]	7
Ala-Ser(PO_3H_2)-**Pro**[d]	8.83	100	100
Ala-Ser(PO_3H_2)-**Pro** -Arg[c]	3.37	270	200
Ser(PO_3H_2)-**Pro** -Arg-NH-Np[e]	5.72	950[i]	n.d.
Ac-Ala-Ala- Ser(PO_3H_2)-**Pro** -Arg -NH-Np[e]	3.30	9500[g]	12000[h]

[a] In 20 mM Tris, pH 7.8. [b] Estimated using the direct assay after pH jump (pH 2.1 → pH 7.8). [c] Estimated using the direct assay after solvent jump from 0.5 M LiCl/TFE into buffer, pH 7.8. [d] Estimated using the direct assay after solvent jump from ethanol into buffer, pH 7.8. [e] Measured at 10 °C with the protease-coupled assay as described in Experimental Procedures. [f] Tested at PPIase concentrations between 50 and 150 nM. [g] This value is very similar to the reported value of Yaffe et al. (*2*). [h] IC_{50} = 55 μM, determined using the protease-coupled assay with the substrate Suc-Ala-Glu-Pro-Phe-NH-Np and nagarse. [i] IC_{50} = 25 μM, determined using the protease-coupled assay with the substrate Suc-Ala-Glu-Pro-Phe-NH-Np and nagarse. nd, not determined.

substrate Ala-Ser(PO_3H_2)-Pro-Arg with a k_{cat}/K_m value of 270 mM^{-1} s^{-1}, indicating a synergism of N- and C-terminal elongation of the Ser(PO_3H_2)-Pro moiety. With a k_{cat}/k_m value of 9300 mM^{-1} s^{-1}, the optimal substrate Ac-Ala-Ala-Ser-(PO_3H_2)-Pro-Arg-NH-Np indicates an extended subsite specificity ranging from at least position P3 to P3′.[3] As depicted in Table 4 the yeast parvulin Ess1 acts in a very similar manner, reflecting the highly conserved catalysis mechanism of the sequence-specific and phosphorylation-dependent PPIases.

To evaluate their affinities to Pin1 active sites, different concentrations of dipeptides were applied in competition with Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NHNp as a substrate in a protease-coupled assay. The resulting K_i values are in the millimolar range (Table 4). The phosphorylation of Ser-Pro led to a 27-fold increase in the affinity to Pin1. It seems that the elongation of the backbone contributes mainly to the transition state binding for catalysis because the poor substrate Ser(PO_3H_2)-Pro-Arg as competitor gave an IC_{50} value only 2-fold higher than that of the good substrate Ser-(PO_3H_2)-Pro-Arg-NHNp.

[3] The subsite nomenclature of Schechter and Berger (*42*) is used to describe the positions relative to the isomerizing bond of the amino acid residues in the peptide.

To convert the Pin1 substrates into specific inhibitors, the prolyl bond carbonyl group or the phosphorylated side chain was changed while leaving other structural elements intact. The oligopeptide conformation is known to tolerate the prolyl peptide bond surrogate Xaa-[CS-N]-Pro, but on the contrary, the catalysis by Cyp18-like PPIases is abolished (*27*). Notably, a conservation of ground state binding affinity of oligopeptides with a changed stereochemistry on the C-α atom at the P1 position to various PPIases has been reported (*28*). However, the enzymatic activities of these PPIases are highly stereospecific. Such conversion prevents activation of the catalytic machinery of human FKBP12, human Cyp18, and *Escherichia coli* Par10. If compared to these enzymes, Pin1 shows phosphorylation dependence (Table 5) as well as stereospecificity. Peptide Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np containing a D-Ser(PO_3H_2) moiety at the P1 subsite failed to undergo catalytic interconversion with up to 40 nM Pin1.

We were able to demonstrate that the incorporation of a D-phosphoserine residue preceding proline yielded a peptide derivative, Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np, with an IC_{50} value of 1 μM (Figure 5, Table 5, entry 8). The binding is phosphorylation-dependent because the control peptide derivative Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np gave

Table 5: IC_{50} Values of Peptide-Derived Inhibitors for Pin1

	Peptides	IC_{50} of Pin1 inhibition
1.	Ser-**Pro**	>> 1mM[a]
2.	Ser-Ψ[CS-N]-**Pro**	>> 1mM[a]
3.	Ser(PO_3H_2)-**Pro**	1.0 ± 0.2 mM
4.	D-Ser(PO_3H_2)-**Pro**	20% inhibition at 1mM
5.	Ac-Ala-Ala-D-Ser-**Pro**-Leu-NH-Np	85 ± 9 μM
6.	Phe-Ser-Ψ[CS-N]-**Pro**-Phe- NH-Np	97 ± 11 μM
7.	Phe-Ser(PO_3H_2)-Ψ[CS-N]-**Pro**-Phe- NH-Np	4.0 ± 0.5 μM
8.	Ac-Ala-Ala-D-Ser(PO_3H_2)-**Pro**-Leu-NH-Np	1.0 ± 0.1 μM
9.	Ac-Ala-Ala-D-Ser(PO_3H_2)-**Pro**-Arg-NH-Np	1.0 ± 0.1 μM[b] / 3.6 ± 0.3 μM[c]

[a] Less than 5% inhibition at 1 mM. [b] Protease-free assay; PPIase domain of Pin1 was used. [c] Protease-free assay; full-length Pin1 was used.

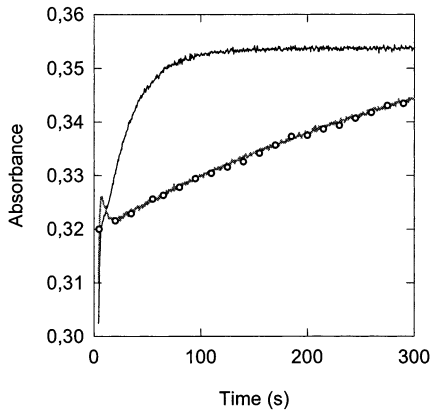

FIGURE 5: Inhibition of Pin1 by Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np. The *cis*/*trans* isomerization of the Pin1 substrate Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np was monitored under 7 °C at 390 nm during an ISP experiment, with trypsin as the auxiliary protease, without addition of enzyme and inhibitor (○) or with 4 nM Pin1 (black) or with 4 nM Pin1 and 15 μM Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np (gray).

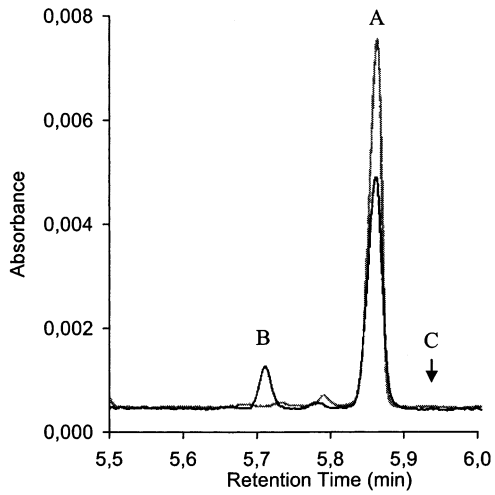

FIGURE 6: Stability of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np against cellular phosphatases. Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np (peak A) was incubated with cell lysate (whole cell lysate of HeLa cells) for 15 min (gray) and 3 h (black) and analyzed with capillary electrophoresis. The capillary thermostating system was kept at 25 °C. Separation buffer was 50 mM phosphate buffer, pH 8.0. Detection was performed at 320 nm. Peak B is a truncated product which contains the 4-nitroanilide moiety and is different from the control peptide Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np. The arrow at peak C refers to the retention time of the control peptide Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np.

approximately 100-fold higher IC_{50} (Table 5, entry 5). The C-terminal leucine residue was designed for estimating the inhibition using Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np as the substrate and trypsin as an isomer-specific protease. Using a protease-free assay, peptide Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np inhibits the Pin1 PPIase domain and full-length Pin1 with IC_{50} values of 1.0 and 3.6 μM, respectively. A very similar situation was found with the incorporation of a thioxylated peptidyl−prolyl bond into a phosphopeptide (Table 5, entry 7). This one-atom substitution represents a minimal deviation of the structure. The IC_{50} of Pin1 inhibition by Phe-Ser(PO_3H_2)-Ψ[CS-N]-Pro-Phe-NH-Np is 4.0 μM.

We investigated the stability of a phospho-D-serine-containing Pin1 inhibitor against cellular phosphatases. Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np was incubated with cell lysate (whole cell lysate of HeLa cells) for 15 min, 1 h, 2 h, and 3 h and analyzed with CE (Figure 6); Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np was used as control. No dephosphorylation of the inhibitor was found. The production of a degraded component during the incubation time could be observed (3.5% after 1 h of incubation at room temperature, 8.5% after 2 h, and 13% after 3 h). The retention time as well as the UV spectrum of this peak indicates that it is a truncated product while the nitroanilide and the phosphate moieties remain intact.

The inhibition of Pin1 by Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np is selective and reversible. No inhibition of Cyp18 and FKBP12 could be detected at inhibitor concentrations as high as 20 μM by using protease-coupled assays of Cyp18 and FKBP12 PPIase activities (*25*). To investigate

the reversibility of Pin1 inhibition by Ac-Ala-Ala-D-Ser-(PO_3H_2)-Pro-Leu-NH-Np, 227 nM Pin1 was incubated with 10 μM inhibitor for 1 min for achieving full inhibition of Pin1. The solution was diluted to a final concentration of 1.77 nM Pin1 and 78 nM inhibitor; 67.7% of Pin1 PPIase activity could be recovered after 5 min of dilution. Furthermore, the inhibition of Pin1 by Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np showed no time dependence. These results indicated that the inhibition of Pin1 by the peptidic mimic is reversible.

DISCUSSION

The crystal structure of Pin1 in complex with Ala-Pro indicates a binding pocket for the Ser(PO_3H_2)-Pro motif (*23*), which is composed of a hydrophobic patch at the active site as well as a basic cluster consisting of Lys-63, Arg-68, and Arg-69. The question raised is whether the dipeptide fulfills the minimal requirements for Pin1 catalysis and if it is able to act as a substrate for Pin1. In this study, we demonstrated that Pin1 catalyzes neither the *cis*/*trans* isomerization of the phosphorylated dipeptide Ser(PO_3H_2)-Pro nor that of the unphosphorylated dipeptides Ser-Pro and Ala-Pro. Instead, the dipeptides act as weak competitive inhibitors for Pin1. Nevertheless, compared with the unphosphorylated dipeptides, a higher binding energy of approximately 10 kJ mol^{-1} of Ser(PO_3H_2)-Pro indicates higher affinity to the protein and is in good agreement with the predicted contact of the phosphate moiety with the basic cluster at the entrance of the active site of Pin1 (*23*). Interestingly, the addition of at least one further peptide bond to Ser(PO_3H_2)-Pro switches the inhibitor to a substrate (Table 4). This observation for Pin1 is very similar to the results of the related PPIase Cyp18, for which a K_i value of 12 mM for Ala-Pro was found (*25*). The additional Ala residue in Ala-Ala-Pro is sufficient to generate a Cyp18 substrate (*25*).

These results suggest an important role for the additional multiple interactions between the extended peptide backbone and the enzymes in catalysis. Hydrophobic grooves involving the catalytic sites are observed in the crystal structures of Cyp18 and FKBP12 as well as in those of Pin1. The extended structure of peptides runs along the groove, as illustrated in the complex of Cyp18 with its ligand, a fragment of HIV-1 Gag protein (*29*), or with its substrate Suc-Ala-Pro-Ala-NH-Np (*30*). In the case of Pin1, the polar residues surrounding the hydrophobic groove might contribute to catalysis through stabilizing the transition state of the peptide undergoing prolyl bond isomerization. Other phosphopeptide binding enzymes have also been shown to make use of ligand backbone interactions, resulting in highly favorable binding (*31*, *32*). On the other hand, a negative effect of the C-terminal and N-terminal charges of the dipeptides sequestered in the hydrophobic groove could also be discussed. Decrease of such influence by backbone elongation and shifting the charges from the prolyl bond results in enhanced ground state binding as well as catalysis.

To investigate the substrate property of the natural peptides discussed above, we developed a sensitive method based on the different absorption coefficients between *cis* and *trans* isomers. This method avoids the incorporation of any artificial chromogenic moieties. The dipeptides Xaa-Pro seem to be the basic unit for investigating prolyl bond isomerization because long-range intramolecular interactions could be completely excluded. The pH-jump method described earlier (*33*−*38*) was used to generate a transient displacement of *cis*/*trans* ratio. Table 1 summarizes the *cis* content of different peptides at neutral and low pH, as either known from the literature (*35*, *36*) or determined by ^{1}H NMR. Generally, the cationic species are favorable in the *trans* conformation, whereas the zwitterionic peptides have a higher *cis* content. The initial and final points of the curves in Figure 1 reflect the different *cis*/*trans* ratio of Ala-Pro at pH 2.1 and 7.8. The resulting rate constant of *cis* → *tran*s isomerization ($k_{c\rightarrow t}$ = 4.2 × 10^{-4} s^{-1} at 10 °C) was confirmed by NMR measurements under similar conditions ($k_{c\rightarrow t}$ = 4.5 × 10^{-4} s^{-1} at 10 °C) (data not shown) and is in good agreement with data from the literature (*39*). Alternatively, solvent jump (*20*) could also induce a redistribution of *cis* and *trans* conformer populations (Figure 3, curve B). For pentapeptide Ac-Ala-Ala-Pro-Ala-Lys-NH_2, the $k_{c\rightarrow t}$ of 3.5 × 10^{-3} s^{-1} at 4 °C is very similar to that of 3.2 × 10^{-3} s^{-1}, determined by NMR spectroscopy at 4 °C (*24*). By this direct assay, we also calculated Arrhenius activation parameters E_A for the *cis* → *trans* isomerization of several dipeptides and Ser-(PO_3H_2)-Pro-Arg (Table 3, Figure 2). This assay also made it possible to investigate the catalytic efficiency of Pin1 to various short peptides (Table 4). Due to the limitation of the assay, extremely poor substrate properties for Pin1 with a k_{cat}/K_m value less than 5 mM^{-1} s^{-1} could not be excluded.

Obviously, elongation of the peptide backbone is necessary but not sufficient for generating a good substrate for Pin1. Further increase of the number of amide bonds affects the catalytic efficiency of Pin1 and Ess1 marginally (Table 4). For instance, addition of one more Ala residue to Suc-Ala-Pro-Phe-NH-Np has no influence on the catalytic efficiency (Table 4). As expected, substrate phosphorylation results in a dramatic increase of k_m/k_{cat} (*2*, *3*) (Table 4). Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np shows a very low *cis*/*trans* interconversion barrier in the presence of Pin1 or Ess1. Therefore, the conclusion can be drawn that the full catalytic potency of Pin1 is achieved only when three requirements for substrate are fulfilled: (1) The pyrrolidine ring of proline has to be present for the interactions with the hydrophobic binding pocket of Pin1. (2) The phosphorylated Ser/Thr residue preceding proline is necessary for high affinity to the active site. (3) A minimum of three amide bonds in the backbone is required to enable productive catalysis by Pin1.

Ess1 is an essential protein in budding yeast. Pin1 is 45% identical to Ess1 and functionally complemented to $ess1^-$ null mutation (*8*). Our results give further evidence that the catalytic mechanism and substrate recognition preferences of Pin1 and Ess1 are well conserved. Nevertheless, a few differences still exist. Compared to Pin1, Ess1 exhibits lower catalytic activities to short substrate, whereas its efficiency toward the optimal substrate is even higher. It seems that the catalytic efficiency of Ess1 depends more restrictively on substrate properties.

A very successful tool in the field of drug design is the utilization of peptide analogues which differ from naturally occurring peptides at certain positions. These analogues are expected to have higher potency and enhanced stability against various enzymatic activities. In principle, there are two routine methods for designing substrate-derived PPIase inhibitors: incorporation of D-amino acids (*28*) and replace-

ment of the prolyl bonds by thioxo amide bonds (*26*).[4]

Like Ser-Pro and Ser(PO_3H_2)-Pro, dipeptide derivatives Ser-Ψ[CS-N]-Pro and D-Ser(PO_3H_2)-Pro were also weak inhibitors for Pin1.[5] Oligopeptides Phe-Ser-Ψ[CS-N]-Pro-Phe-NH-Np and Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np showed higher affinity to Pin1 than the dipeptide derivatives. Obviously, extended binding sites were also beneficial for high affinity.

As proof of the concept concerning inhibitor design, we chose the best substrate for Pin1 in this study, Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np, for a single substitution in the position preceding proline by the D-amino acid. As expected, the *cis* → *trans* isomerization of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Arg-NH-Np is not catalyzed by Pin1 even at high enzyme concentration, as measured by a protease-coupled assay with trypsin as the auxiliary protease. To evaluate inhibition and to prevent it itself from being cleaved by trypsin in the assay, a protease-free assay was applied to measure its ground state binding affinities to the Pin1 PPIase domain as well as to full-length Pin1. The peptide mimic exhibits a strong inhibition to the enzyme and its catalytic domain (Table 5). Thioxo amide bond substitution as well as D-amino acid substitution was applied to sequences that cannot be cleaved by trypsin, and therefore the protease-coupled assays were performed. As shown in Table 5, both compounds exhibit remarkable inhibition of Pin1.

By replacing L-Ser with D-Ser in peptides, substrate specificity studies have shown that the nonnatural peptides cannot be phosphorylated as efficiently by kinases as their parent sequences (*40*, *41*). We suggested a similar retardation of phosphatase-catalyzed dephosphorylation of phosphorylated D-amino acid in peptides. In our study, we examined the stability of Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np in whole cell lysate from HeLa cells. As analyzed by CE (Figure 6) with Ac-Ala-Ala-D-Ser-Pro-Leu-NH-Np as the reference, the phosphopeptide exhibits remarkable stability against cellular phosphatases. Only some truncated products were discovered after long incubation, but no dephosphorylation was observed. Thus the substitution of a D-phosphoserine for an L-phosphoserine has two different effects on the character of the peptide: it changes a good Pin1 substrate into a potent inhibitor. On the other hand, it enhances the stability of the compound against cellular phosphatase activity.

In summary, on the basis of the different UV−visible absorption spectra in the amide bond region for the *cis* and *trans* isomers of prolyl bonds, we were able to follow the isomerization without introducing any artificial chromogenic functionalities. Using this assay we demonstrated that dipeptides, regardless of possessing the critical elements of Pin1 recognition, the prolyl bond and the phosphorylated side chain, are not substrates but competitive inhibitors for Pin1. Both phosphorylated Ser/Thr side chains preceding proline and the extension of the backbone on the N- or C-terminus are required for Pin1 catalysis. Fulfilling the same requirements, we were able to design substrate-derived inhibitors for Pin1 with affinities in the low micromolar range by substitution of phospho-D-amino acid or thioxo peptide bonds. The inhibition of Pin1 is reversible and specific. Furthermore, Ac-Ala-Ala-D-Ser(PO_3H_2)-Pro-Leu-NH-Np exhibits marked stability against cellular phosphatase activity.

[4] To introduce C═S, the prefix "thiono" has frequently been used in place of "thioxo". The IUPAC nomenclature committee nevertheless recommends the use of thioxo.

[5] Alterations of a peptide bond are represented by the Ψ nomenclature system. A character Ψ is followed by the structure of the new bond in brackets. The nomenclature of the compounds is in accordance with the recommendations of the IUPAC−IUB Commission on Biochemical Nomenclature (*43*).

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