# Thermodynamics of Phosphopeptide Binding to the Human Peptidyl Prolyl *cis/trans* Isomerase Pin1

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ABSTRACT: Proteins containing phosphorylated Ser/Thr-Pro motifs play key roles in numerous regulatory processes in the cell. The peptidyl prolyl *cis/trans* isomerase Pin1 specifically catalyzes the conformational transition of phosphorylated Ser/Thr-Pro motifs. Here we report the direct analysis of the thermodynamic properties of the interaction of the PPIase Pin1 with its substrate−analogue inhibitor Ac-Phe-D-Thr-($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ specifically targeted to the PPIase active site based on the combination of isothermal titration calorimetry and studies on inhibition of enzymatic activity of wt Pin1 and active site variants. Determination of the thermodynamic parameters revealed an enthalpically and entropically favored interaction characterized by binding enthalpy $\Delta H_{ITC}$ of $-6.3 \pm 0.1$ kcal mol$^{-1}$ and a $T\Delta S_{ITC}$ of $4.1 \pm 0.1$ kcal mol$^{-1}$. The resulting dissociation constant $K_D$ for binding of the peptidic inhibitor with $1.8 \times 10^{-8}$ M resembles the dissociation constant of a Pin1 substrate in the transition state, suggesting a transition state analogue conformation of the bound inhibitor. The strongly decreased affinity of Pin1 for ligand at increasing ionic strength implicates that the potential of bidentate binding of a substrate protein by the PPIase and the WW domain of Pin1 may be required to deploy improved efficiency and specificity of Pin1 under conditions of physiological ionic strength.

The human peptidyl-prolyl *cis*/*trans* isomerase (PPIase, EC. 5.2.1.8.)[1] Pin1 shares the ability of all PPIases to accelerate the *cis/trans* isomerization of imidic peptide bonds preceding proline (*1*, *2*). However, when assayed with phosphopeptides in vitro, Pin1 showed a unique preference for Ser($PO_3H_2$)/Thr($PO_3H_2$)-Pro substrate motifs, which distinguishes it from other PPIases (*2*, *3*). Pin1 consists of an N-terminal type-IV WW domain, a common protein−protein interaction module linked to a C-terminal PPIase domain tethered by a flexible segment. While both domains preferentially bind Ser($PO_3H_2$)/Thr($PO_3H_2$)-Pro containing peptide stretches, only the PPIase domain demonstrates catalytic activity (*2*, *4*, *5*).

Proline-directed phosphorylation is a frequent event in cell cycle progression, transcription, translation, endocytosis, and apoptosis. It modulates the activities of proteins involved, and can be found in a large variety of proteins. As suggested by the multitude of substrate proteins and interaction partners already shown to be targeted by Pin1, like, for example, Cdc25, c-Myc, tau, p53, and RNA polymerase II (*6*−*10*), this enzyme was found to play regulatory roles in many critical cellular functions. Pin1 overexpression causes G2/M arrest and delayed entry into mitosis (*11*−*13*). The depletion of Pin1 from HeLa cells leads to mitotic arrest (*11*), and mice lacking Pin1 are viable but develop cell-proliferative abnormalities (*14*, *15*). Interestingly, the depletion of Pin1 in athymic mice inhibits tumor growth and angiogenesis (*16*). Pin1 is generally overexpressed in human cancers and has been linked to oncogenesis (*17*−*19*) thus forming a potential target for anticancer drugs (*20*).

Furthermore, Pin1 is the first protein whose depletion has been found to cause age-dependent neurodegeneration and tau pathologies (*21*). Recently, Pin1 was described to be a key mediator of the production of antiapoptotic cytokine granulocyte-macrophage colony-stimulating factor, which is involved in eosinophil activation and thus involved in the development of active asthma (*22*). The use of Pin1 variants with impaired PPIase activity showed the importance of the PPIase activity of Pin1 for the cellular functions of the enzyme (*23*).

In vitro, considerable catalytic activity of Pin1 can be achieved, provided the substrate contains a minimum of three backbone amide bonds, a prolyl residue for the interaction with the hydrophobic binding pocket formed by Pin1 residues Leu122, Met130, and Phe134, and a Ser($PO_3H_2$)/(Thr($PO_3H_2$) residue preceding proline (*24*). Most efficient catalysis is observed at pH values where the phosphorylated side chain is in its dianionic form. In the crystal structure of the Pin1/Ala-Pro complex, a sulfate ion was found inside the substrate binding pocket of the PPIase domain complexed by the basic side chains of Lys63, Arg68, and Arg69 (*25*).

This basic cluster forms the recognition site for the phosphate group of Pin1 substrates, because substitution of alanine for the two arginine residues Arg68 and Arg69 reduces the catalytic efficiency for phosphorylated substrate peptides to the low level of unphosphorylated peptide substrates. When compared to monophosphorylated substrates, those containing two Ser($PO_3H_2$)/Thr($PO_3H_2$)-Pro

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[1] Abbreviations: ITC, isothermal titration calorimetry; Pin1ΔWW, Pin1 lacking the WW domain (Pin1 AS 45-163); pNA, 4-nitroanilide; PPIase, peptidyl prolyl *cis*/*trans* isomerase; WT, wild type.

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motifs showed increased affinity to both the WW and the PPIase domain of Pin1 but were found to be associated with a markedly decreased effectiveness toward Pin1 catalysis (*5*, *26*, *27*).

Thus, we have come to ask whether the catalytic machinery of Pin1 has evolved to facilitate the process of Ser-($PO_3H_2$)/Thr($PO_3H_2$)-Pro- isomerization by synergistic group contributions of the phosphorylated substrates. Here we made use of the substrate-derived ligand Ac-Phe-D-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ that allowed the full range of thermodynamic investigations of stable Pin1-phosphopeptide complexes using isothermal titration calorimetry and enzyme activity assays. Ligand binding completely discriminated the WW domain from the PPIase site that is competitively inhibited with a $K_I$ value of 18.3 ± 1.3 nM (*28*). Linear correlations of $\log(k_{cat}/K_M)$ for substrate turnover versus $pK_I$ of the inhibitor at different ionic strength have slopes of approximately 0.5, which suggest that the D-Thr($PO_3H_2$)-Pip moiety binds to Pin1 in a manner that structurally resembles transition states of catalyzed prolyl bond rotation.

The loss of Pin1-phosphopeptide complex stability at elevated ionic strength is enthalpically driven and mirrors the reduced catalytic efficacy of the enzyme examined under similar conditions. Comparison of the salt dependencies of catalytic parameters obtained from other human PPIases revealed a reduced catalytic selectivity of Pin1 under cellular conditions. We also tested whether the presence of the WW domain affects structural parameters of the Pin1 complexes with monophosphorylated substrates. Analysis of the type and the magnitude of the changes in solvent-accessible areas, $\Delta ASA_{apolar}$ and $\Delta ASA_{polar}$, did not reveal a marked communication between both domains for ligand binding.

## EXPERIMENTAL PROCEDURES

*Chemicals.* Buffers were purchased from AppliChem (Darmstadt, Germany) or Merck (Darmstadt, Germany). Yeast extract and Peptone were purchased from Serva (Heidelberg, Germany). Sodium phosphate was purchased from Merck. All other chemicals were purchased from Sigma (Munich, Germany) and of the highest purity available.

*Peptides.* Ac-Phe-D-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ (**1**), Ac-Phe-L-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ (**2**), Ac-Phe-D-Glu-Pip-Nal-Gln-$NH_2$ (**3**), Ac-Phe-L-Glu-Pip-Nal-Gln-$NH_2$ (**4**), and the chromogenic Pin1 substrate Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-pNA were synthesized by Fmoc chemistry as has already been described (*3*, *28*). Suc-Ala-Glu-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA were purchased from Bachem (Heidelberg, Germany).

*Enzymes.* The DNA fragments encoding Pin1 or Pin1ΔWW variant were amplified from Pin1 coding sequence in pET28a (kindly provided by K. P. Lu) and inserted in the pET28a vector using NcoI and HindIII to obtain wild-type Pin1 or the Pin1ΔWW variant without tags, respectively. The point mutations Arg68Ala, Arg69Ala, and Arg68/69Ala were introduced into the expression cassette using Quick-Change site-directed mutagenesis (Stratagene). Correct DNA sequences were confirmed for all variants by automated sequencing.

*Protein Expression and Purification.* Competent *Escherichia coli* BL21(DE3) cells were transformed with 100 ng of plasmid DNA containing either WT Pin1 or Pin1ΔWW variant or mutant DNA. Protein overexpression was achieved in LB medium, at an OD value of 0.6, after induction with 1 mM IPTG for 4 h at 310 K. Cell pellets were collected by centrifugation for 15 min at 4000 RCF. After cell lyses via French Press in 10 mM HEPES buffer pH 7.2, 1 mM DTT recombinant Pin1 or Pin1ΔWW variant were purified. The soluble protein fraction was diluted in 50 mL of the same buffer and separated from the cell debris by ultracentrifugation at 96000 RCF and 277 K using a Beckmann LE-80K ultracentrifuge. The crude extract was applied to a DEAE anion exchange chromatography column at a flow rate of 3 mL/min. The unbound fraction containing the desired protein was then applied to a Q-Sepharose cation exchange chromatography column and eluted with an NaCl gradient (0−1 M in 10 mM HEPES buffer pH 7.2, 1 mM DTT) at a flow rate of 1.5 mL/min. Fractions containing the desired protein were concentrated and finally purified by size exclusion chromatography in 10 mM HEPES buffer pH 7.8, 150 mM $MgCl_2$, 1.5 mM KCl, 1 mM DTT using a Sephadex75 column at a flow rate of 0.8 mL/min. The purification progress was analyzed after each step by SDS PAGE and revealed a single band after the last purification step. N-terminal sequencing and mass spectrometry were used to confirm the identity and correct mass of all Pin1 variants.

*PPIase Activity Assay and Inhibition Studies.* PPIase activity assays were performed at 283 K in quartz cuvettes with a path length of 1 cm under vigorous stirring with a Hewlett-Packard 8453A UV/vis spectrophotometer in 35 mM HEPES buffer at pH 7.8. WT Pin1 and Pin1 variants were used at concentrations between 4.6 nM and 30 nM. Enzyme activities of Pin1 and the variants toward the substrates Suc-Ala-Glu-Pro-Phe-pNA or Suc-Ala-Ala-Pro-Phe-pNA were measured using the protease free assay according to Janowski et al. (*29*). A 30 mM stock solution of the substrate in 0.5 M LiCl/TFE (anhydrous) was prepared fresh before the measurement (60 μM final substrate concentration). Prior to every measurement, all components except substrate were incubated for 300 s at 283 K. The measurement was started upon substrate addition, and the *cis*/*trans* isomerization kinetics of the substrate was followed at 330 nm. Enzyme activities in the substrate Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-pNA were measured using the protease-coupled PPIase assay according to Fischer et al. (*30*). The substrate was dissolved at 20 mg/mL in DMSO. Prior to every measurement, substrate and enzyme were incubated for 300 s at 283 K. The measurement was started after the addition of trypsin (250 μg/mL final concentration), and the *cis*/*trans* isomerization kinetics of the substrate was followed at 390 nm.

*Isothermal Titration Calorimetry (ITC).* Titration experiments were performed with a MicroCal VP-ITC at the indicated temperatures. To ensure proper function, the instrument was heat-pulse-calibrated. Protein samples were extensively dialyzed against 35 mM HEPES buffer at pH 7.8. Inhibitor stock solutions (20 mM) were prepared in deionized water and diluted prior to the experiment to an appropriate concentration into the same batch of buffer as used for dialysis. All solutions used were degassed before filling the sample cell and syringe. During a typical titration experiment, 300 μL of a 100−500 μM inhibitor solution were added to 1.4 mL of 10−20 μM protein in 15 μL steps. The ITC stirring speed was set to 310 rpm; the feedback gain

mode was set to high. Since the initial injection generally delivers inaccurate data, only 2 $\mu$L were injected in this step and the recorded data discarded. As a control experiment, the inhibitor was titrated into buffer, and the measured heat of dilution was used to mathematically correct the experimental data. For pH dependencies, a mixture of 25 mM MES, 25 mM sodium acetate, 50 mM Tris, and 150 mM NaCl was used as buffer system in order to maintain a constant buffer composition for experiments performed at different pH values (*31*). The collected data were analyzed using the program "Origin" (MicroCal). Binding isotherms were fitted according to a one binding site model. Errors correspond to the SD of the nonlinear least-squares fit of the data points of the titration curve.

*Determination of the Solvent Accessible Surface Areas (ASA).* According to methods introduced by Xie and Freire (*32*), the thermodynamic parameters $\Delta C_{Ptheor}$ and $\Delta H$ (333 K) can be calculated based on the molecular structure of a protein ligand complex. The parameters were calculated according to eqs 1 and 2. $\Delta ASA_{polar}$ and $\Delta ASA_{apolar}$ represent changes of the solvent accessible surface area on protein and ligand during the complex formation. The parameters $a$ = 0.45 cal $K^{-1}$ $mol^{-1}$ $A^{-2}$, $b = -0.26$ cal $K^{-1}$ $mol^{-1}$ $A^{-2}$; $c$ = 31.4 cal $K^{-1}$ $mol^{-1}$ $A^{-2}$, and $d$ = 8.44 cal $K^{-1}$ $mol^{-1}$ $A^{-2}$ were taken from the literature (*32*).

$$\Delta C_{Ptheor} = a\Delta ASA_{apolar} + b\Delta ASA_{polar} \quad (1)$$

$$\Delta H(333\ K) = c\Delta ASA_{polar} - d\Delta ASA_{apolar} \quad (2)$$

## RESULTS

*Thermodynamics of Binding of a Substrate−Analogue Inhibitor.* Isothermal titration calorimetry was used to determine the thermodynamic parameters of the association reaction of the inhibitor Ac-Phe-D-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ (**1**) with full-length recombinant Pin1 and the WW domain lacking Pin1, Pin1ΔWW variant. The titration of 10 $\mu$M Pin1 with 200 $\mu$M **1** at 293 K revealed a binding enthalpy $\Delta H_{ITC}$ of $-6.3 \pm 0.02$ kcal $mol^{-1}$, a $T\Delta S_{ITC}$ of 4.1 $\pm$ 0.01 kcal $mol^{-1}$, and a $\Delta G_{ITC}$ of $-10.4 \pm 1.1$ kcal $mol^{-1}$, which results in an association constant $K_A$ of (5.40 $\pm$ 0.6) $\times$ $10^7$ $M^{-1}$. This value agrees well with the $1/K_I$ value of (4.90 $\pm$ 1.0) $\times$ $10^7$ $M^{-1}$ as determined by measuring the concentration-dependent inhibition of Pin1 activity by **1**. Values for $T\Delta S_{ITC}$ and $\Delta G_{ITC}$ were obtained using the Gibbs−Helmholtz equation

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

and

$$\Delta G = -RT\ln(K_D) \quad (4)$$

, respectively. The stoichiometry factor $N$ of 1.12 indicates an equimolar interaction such as the formation of a 1:1 complex. A binding enthalpy $\Delta H_{ITC}$ of $-6.3 \pm 0.1$ kcal $mol^{-1}$, a $T\Delta S_{ITC}$ of 4.0 $\pm$ 0.07 kcal $mol^{-1}$, and a $\Delta G_{ITC}$ of $-10.3 \pm 3.0$ kcal $mol^{-1}$, which results in a $K_A$ value of (4.80 $\pm$ 1.4) $\times$ $10^7$ $M^{-1}$ with a stoichiometric factor $N$ of 1.01 was determined for the association of **1** and 10 $\mu$M Pin1ΔWW variant, indicating that the influence of the WW domain on inhibitor binding is negligible. This feature forms a prerequisite for the evaluation of the ligand interacting properties of the PPIase domain of Pin1.

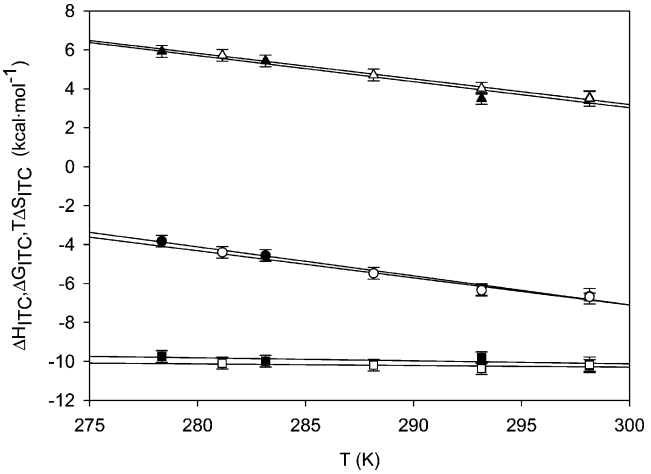


FIGURE 1: Temperature dependence of $\Delta H_{ITC}$ (circles), $\Delta G_{ITC}$ (squares), and $T\Delta S_{ITC}$ (triangles) for binding of **1** to Pin1 (closed symbols) or Pin1ΔWW (open symbols). The measurements were performed in 35 mM HEPES, pH 7.8, at 293 K. Error bars represent the SD of the nonlinear least-squares fit of the data points of the titration curve. The drawn line represents the linear regression of the data.

The thermodynamic parameters of the interaction show that the association reaction is enthalpically and entropically driven. The enthalpic contribution reflects the favorable change in noncovalent bond energy occurring during the interaction. The positive entropic contribution hints as a burial of solvent-accessible surface area on binding, since the release of ordered water molecules often contributes extensively and positively to the entropy of an interaction (*33*).

The change in hydrophobic surface area of a protein upon binding to its ligand can be assessed by the change in heat capacity, which can be determined from binding enthalpy at different temperatures. Therefore, binding of **1** to Pin1 and Pin1ΔWW were analyzed by a series of ITC experiments at different temperatures ranging from 278 to 298 K. In both cases, the values of $\Delta H_{ITC}$ and $T\Delta S_{ITC}$ are linearly dependent on temperature, while the $\Delta G_{ITC}$ values are almost insensitive to increasing temperatures (Figure 1). The measurements were performed in 35 mM HEPES buffer pH 7.8. The ionic strength of the buffer did not change significantly due to the temperature-sensitive p$K_a$ value of HEPES buffer in the applied temperature range ($\Delta pK_a/dT = -0.014$ $K^{-1}$) and thus was not included in our analyses. A plot of the temperature-dependent $\Delta H_{ITC}$ vs $T\Delta S_{ITC}$ values (data not shown) reveals a slope of 1.07 for both enzymes. A slope near unity is often observed for protein/ligand interaction indicating enthalpy−entropy compensation (*34*).

According to eq 5, linear regression of the temperature-dependent $\Delta H_{ITC}$ values revealed heat capacity changes $\Delta C_P$ of $-148 \pm 13$ cal $mol^{-1}$ $K^{-1}$ and $-139 \pm 14$ cal $mol^{-1}$ $K^{-1}$ for the interaction of **1** with Pin1 and Pin1ΔWW, respectively.

$$\Delta C_P = \left(\frac{\delta \Delta H}{\delta T}\right)_P \quad (5)$$

Calculation of the solvent-accessible area ΔASA according to Xie and Freire (*32*) gave $\Delta ASA_{polar}$ of $-550$ $Å^2$ and

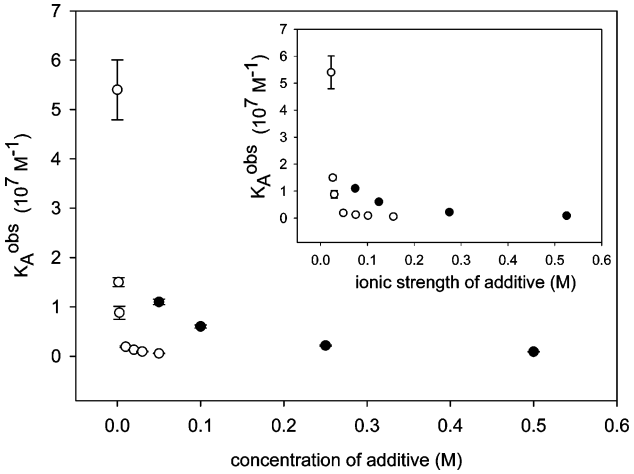


FIGURE 2: Association constants ($K_A$) for the interaction of Pin1 and **1** acquired in 35 mM HEPES, pH 7.8, 293 K, in the presence of NaCl (closed circle) or $NaH_2PO_4/Na_2HPO_4$ (open circle). The inset shows $K_A$ dependent on the ionic strength in 35 mM HEPES, pH 7.8, at the additional presence of NaCl (closed circle) or $NaH_2PO_4/Na_2HPO_4$ (open circle). Error bars represent the SD of the nonlinear least-squares fit of the data points of the titration curve.

$\Delta ASA_{apolar}$ of $-646$ Å$^2$ for Pin1 and $\Delta ASA_{polar}$ of $-523$ Å$^2$ and $\Delta ASA_{apolar}$ of $-611$ Å$^2$ for Pin1ΔWW.

*Influence of Ionic Strength on the Pin1/Ligand Interaction.* Inorganic phosphate has been shown to inhibit catalysis of the Glu-Pro bond isomerization by Pin1. However, the strict dependence of PPIase activity on ionic strength upon addition of the monobasic chloride ion suggests that the electrostatic situation in the Pin1 active site rather than a specific interaction with inorganic phosphate is critical for Pin1-substrate interactions (*3*). To dissect a specific phosphate-mediated interaction from more general contributions of ionic strength, we examined the influence of increasing concentrations of chloride and inorganic phosphate on the thermodynamic parameters of the Pin1/**1** association. Pin1 at a concentration of 10 $\mu$M was titrated with **1** in 35 mM HEPES buffer pH 7.8 in the additional presence of different concentrations of NaCl (0−500 mM) or $NaH_2PO_4/Na_2HPO_4$ (0−50 mM).

The affinity of the inhibitor to Pin1 is strongly decreased with increasing ionic strength (Figure 2). At very high concentrations of chloride or inorganic phosphate, the association constant $K_A$ was decreased to about 1.5% of the original value in 35 mM HEPES buffer. A 50% reduction of $K_A$ was observed in the presence of 15 mM NaCl (ionic strength of the additive is 0.015). For phosphate ions this value was calculated as 0.5 mM (ionic strength of the additive is 0.0013).

Apparently the decreased affinity of **1** to Pin1 in the presence of chloride ions is largely due to the 2.6 kcal mol$^{-1}$ more positive $\Delta H_{ITC}$, while the change of the entropic contribution with $-0.2$ kcal mol$^{-1}$ is low (Table 1). Likewise, the lowered affinity of **1** in the presence of phosphate ions is caused by a 4.5 kcal mol$^{-1}$ more positive $\Delta H_{ITC}$, which is partially compensated by a 1.8 kcal mol$^{-1}$ more favorable $T\Delta S_{ITC}$. However, the change of $T\Delta S_{ITC}$ for binding of **1** to Pin1 in the presence of increasing concentrations of inorganic phosphate is characterized by a large increase of $T\Delta S_{ITC}$ at low concentrations of phosphate with a maximal $T\Delta S_{ITC}$ between 1.5 and 2.5 mM followed by a slight decrease at higher concentrations, pointing to a more complicated picture involving at least two different counteracting phosphate effects.

Table 1: Effects of Chloride and Inorganic Phosphate on Thermodynamic Parameters of the Interaction of Pin1 and **1**[a]

| $Na_2HPO_4$/$NaH_2PO_4$ (mM) | $\Delta H_{ITC}$ (kcal mol$^{-1}$) | $T\Delta S_{ITC}$ (kcal mol$^{-1}$) | $K_A$ ($10^7$ M$^{-1}$) |
|---|---|---|---|
| 0 | $-6.3 \pm 0.02$ | $4.1 \pm 0.01$ | $5.40 \pm 0.60$ |
| 1.5 | $-2.7 \pm 0.01$ | $6.9 \pm 0.02$ | $1.50 \pm 0.01$ |
| 2.5 | $-2.4 \pm 0.03$ | $6.9 \pm 0.07$ | $0.88 \pm 0.13$ |
| 10 | $-2.0 \pm 0.02$ | $6.4 \pm 0.06$ | $0.19 \pm 0.02$ |
| 20 | $-2.1 \pm 0.01$ | $6.1 \pm 0.03$ | $0.13 \pm 0.01$ |
| 30 | $-2.0 \pm 0.02$ | $6.0 \pm 0.05$ | $0.09 \pm 0.07$ |
| 50 | $-1.8 \pm 0.04$ | $5.9 \pm 0.11$ | $0.06 \pm 0.02$ |
| NaCl (mM) | $\Delta H_{ITC}$ (kcal mol$^{-1}$) | $T\Delta S_{ITC}$ (kcal mol$^{-1}$) | $K_A$ ($10^7$ M$^{-1}$) |
| 0 | $-6.3 \pm 0.02$ | $4.1 \pm 0.01$ | $5.40 \pm 0.60$ |
| 50 | $-4.8 \pm 0.02$ | $4.4 \pm 0.02$ | $1.10 \pm 0.06$ |
| 100 | $-4.4 \pm 0.02$ | $4.6 \pm 0.02$ | $0.60 \pm 0.03$ |
| 250 | $-4.2 \pm 0.03$ | $4.3 \pm 0.02$ | $0.22 \pm 0.01$ |
| 500 | $-3.7 \pm 0.03$ | $4.3 \pm 0.02$ | $0.09 \pm 0.01$ |

[a] Data were obtained by titration of a 100−500 $\mu$M solution of **1** to 20 $\mu$M Pin1 in 35mM HEPES buffer at pH 7.8 at 293 K in the additional presence of the additives in the concentrations depicted.

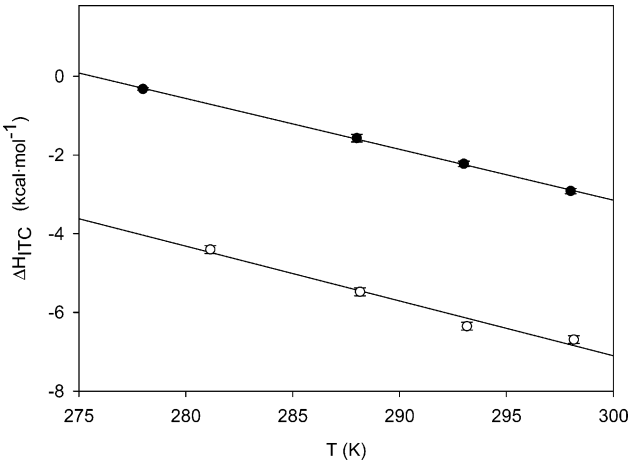


FIGURE 3: Temperature dependence of $\Delta H_{ITC}$ for the Pin1 ΔWW/**1** interaction in 35 mM HEPES buffer at pH 7.8 in the presence (closed circle) or absence (open circle) of 30 mM $NaH_2PO_4/Na_2HPO_4$. Error bars represent the SD of the nonlinear least-squares fit of the data points of the titration curve. The drawn line represents the linear regression of the data resulting in $\Delta C_P$ values for the interaction of Pin1 ΔWW and **1** of $-139 \pm 14$ cal mol$^1$ K$^{-1}$ in the absence and $-129 \pm 4$ cal mol$^{-1}$ K$^{-1}$ in the presence of inorganic phosphate.

While $\Delta H_{ITC}$ values are increased when phosphate is present, there is only a small effect on $\Delta C_P$. The Pin1ΔWW/inhibitor association was measured at different temperatures between 278 and 298 K in the presence and absence of phosphate (Figure 3). Linear regression of the observed binding enthalpies revealed $\Delta C_P$ values of $-139 \pm 14$ cal mol$^{-1}$ K$^{-1}$ for Pin1ΔWW in the absence of phosphate and $-129 \pm 4$ cal mol$^{-1}$ K$^{-1}$ when 30 mM phosphate is present.

Direct measurements of the PPIase activity of Pin1 in the protease coupled assay with Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-pNA as the substrate in the presence of sodium chloride or phosphate showed that inorganic phosphate inhibits Pin1 with an apparent inhibition constant $K_I$ of $1.7 \pm 1.5$ mM

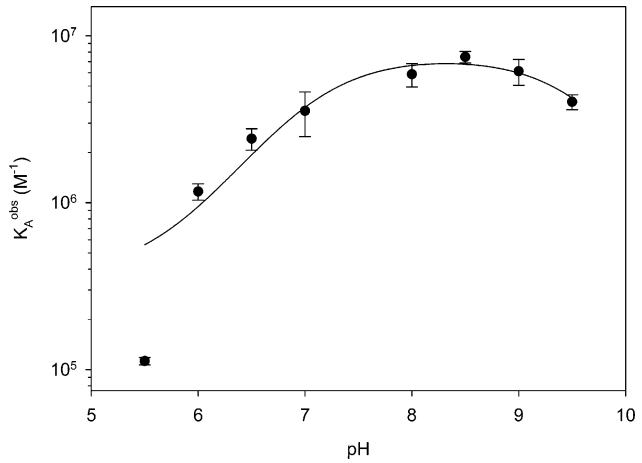


FIGURE 4: pH dependence of the association constant $K_A$ for the Pin1/**1** interaction at 293 K. To maintain a constant buffer composition at different pH values, a mixture of 25 mM MES, 25 mM sodium acetate, 50 mM Tris, and 150 mM NaCl was used. The solid line represents the fit of eq 6 to the measured data, resulting in two p$K_A$ values for ligand and enzyme in the bound (p$K_{ab1}$= 5.74 and p$K_{ab2} \gg 9$) and unbound form (p$K_{au1}$ = 7.05 and p$K_{au2}$ = 9.61) each.

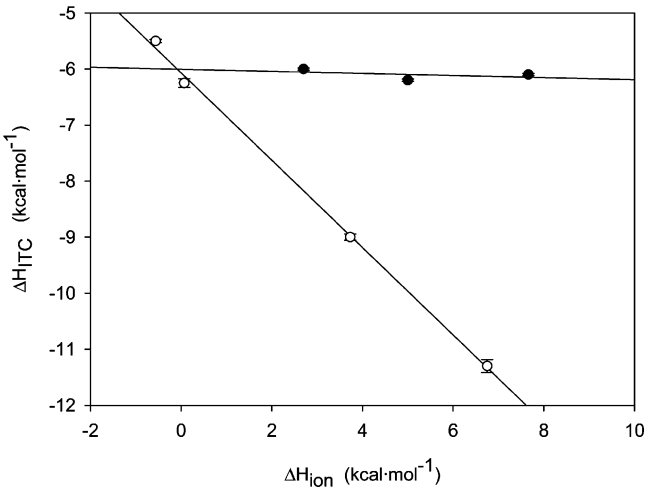


FIGURE 5: Binding enthalpies for the interaction of Pin1 with **1** at pH 5.5 (open circle) and pH 7.5 (closed circle). At both pH values buffer systems with different ionization enthalpies $\Delta H_{ion}$ were used. Cacodylat: $\Delta H_{ion} = -0.56$ kcal mol$^{-1}$. Acetate: $\Delta H_{ion} = 0.07$ kcal mol$^{-1}$. MES: $\Delta H_{ion} = 3.73$ kcal mol$^{-1}$. Bis-Tris: $\Delta H_{ion} = 6.75$ kcal mol$^{-1}$ at pH 5.5. PIPES: $\Delta H_{ion} = 2.76$ kcal mol$^{-1}$. HEPES: $\Delta H_{ion} = 5.0$ kcal mol$^{-1}$. Tricine: $\Delta H_{ion} = 7.66$ kcal mol$^{-1}$ at pH 7.5. The observed binding enthalpies ($\Delta H_{ITC}$) were plotted against the buffer ionization enthalpy ($\Delta H_{ion}$).

(ionic strength of the additive is 0.0044), which corresponds well with a $K_D$ of 2 mM obtained by fluorescence titration (*35*). In the presence of chloride, an apparent $K_I$ value of 45.1 ± 7.3 mM (ionic strength of the additive is 0.045) was observed similar to the already reported value of 50 mM for the inhibition of the catalysis of the Glu-Pro isomerization by Pin1 (*3*). The similarity of the magnitude of the observed influence of the ions on binding of the inhibitor and the catalysis of a substrate suggests that (i) an influence on binding rather than catalysis is observed and (ii) binding of **1** seems to mimic productive interaction quite well.

*Protons in the Pin1/Ligand Interaction.* Since the enzyme activity of Pin1 toward phosphorylated substrates strongly depends on the ionization state of the phosphorylated side chain (*3*), we wanted to determine the influence of pH on the association between Pin1 and a phosphorylated ligand. Isothermal titration experiments using Pin1 and **1** were performed at pH values between 5.5 and 9.5. For these experiments a three-component buffer mixture was used that allows maintenance of constant ionic strength of $I = 0.2$ throughout the investigated pH range (*31*). The observed association constants ($K_A^{obs}$) were plotted against pH revealing a curve with maximal $K_A^{obs}$ between pH 8.0 and 9.0 and a strongly decreased binding affinity under acidic conditions (Figure 4).

Binding constants are only dependent on pH, if complex formation leads to changes in the dissociation behavior of the interaction partners. Because of the slight decrease of $K_A^{obs}$ at high pH, the data were fitted using a model, which accounts for two ionizable groups, both changing their p$K_a$ values upon binding (eq 6).

$$K_A^{obs} = K_A \frac{1 + 10^{pK_{ab2}-pH} + 10^{pH-pK_{ab1}}}{1 + 10^{pK_{au2}-pH} + 10^{pH-pKau1}} \qquad (6)$$

The fit resulted in two p$K_a$ values in the unbound state (p$K_{au1}$ = 7.05 and p$K_{au2}$ = 9.61) and two p$K_a$ values after complex formation (p$K_{ab1}$ = 5.74 and p$K_{ab2} \gg 9$). A value of 7.50 × $10^6$ $M^{-1}$ was determined for the pH-independent association constant ($K_A$). Apparently one group has a p$K_a$ value of about 7.05 in the free state and 5.74 in the enzyme/ligand complex. Efficient catalysis by Pin1 of the isomerization of phosphorylated Ser/Thr-Pro bonds was already shown to be specific to the dianionic form of the side chain phosphate (*3*). Therefore we assume that the phosphoester moiety of **1** is likely to be responsible for the thermodynamically significant dissociation step at lower pH and will be favorably bound to Pin1 in its dianionic form. The $K_A$ value at pH 5.5 does not fit well to eq 6. Apparently, the dissociation behavior is more complex in this pH range. The decrease of the association constant at pH values >9 might indicate an involvement of a lysyl or cysteinyl residue in complex formation.

If ionizable groups undergo changes of their p$K_a$ upon complex formation, protons will be exchanged with the buffer system. To elucidate the proton balance during inhibitor binding, Pin1 was titrated with **1** using various buffers of different ionization enthalpies $\Delta H_{ion}$ at pH 5.5 and pH 7.5. According to eq 7, the observed binding enthalpy ($\Delta H_{ITC}$) is a function of the buffer-independent binding enthalpy ($\Delta H_{bind}$) and the buffer ionization enthalpy ($\Delta H_{ion}$). The factor $n$ refers to the number of protons that are taken up ($n > 0$) or released ($n < 0$) by the protein/ligand complexation.

$$\Delta H_{ITC} = \Delta H_{bind} + n\Delta H_{ion} \qquad (7)$$

The experimentally observed enthalpy changes ($\Delta H_{ITC}$) were plotted against the ionization enthalpy of the buffer ($\Delta H_{ion}$) (Figure 5). The slope of the linear regression of the data revealed $n$-values of −0.77 ± 0.02 at pH 5.5 and −0.02 ± 0.03 at pH 7.5. This indicates that at pH 5.5, 0.77 protons are transferred to the buffer during binding. A decrease of one or more p$K_a$ values of ionizable groups corresponding to donor proton groups of the ligand and/or Pin1 must take place. The virtual lack of net proton transfer at pH 7.5 points to a complete deprotonation of the ionizable groups relevant

Table 2: Catalytic Efficiency ($k_{cat}/K_M$) of Pin1 and Pin1 Variants toward Various Substrates

| | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) | | | |
|---|---|---|---|---|
| Xaa | wt | Arg68Ala | Arg69Ala | Arg68/69Ala |
| L-Ser($PO_3H_2$)[a] | $(1.31 \pm 0.21) \times 10^7$ | $(2.33 \pm 0.04) \times 10^6$ | $(1.26 \pm 0.17) \times 10^6$ | $(5.89 \pm 1.40) \times 10^4$ |
| L-Glu[b] | $(3.53 \pm 0.43) \times 10^6$ | $(3.11 \pm 0.09) \times 10^6$ | $(5.96 \pm 0.47) \times 10^5$ | $(2.71 \pm 0.51) \times 10^4$ |
| L-Ala[b] | $(4.84 \pm 0.39) \times 10^4$ | $(2.89 \pm 0.15) \times 10^4$ | $(4.29 \pm 0.60) \times 10^4$ | $(1.72 \pm 0.18) \times 10^4$ |

[a] Since the phosphorylated substrate does not allow measurement with the protease-free assay, measurements with Ac-Ala-Ala-Ser($PO_3H_2$)-Pro-Arg-pNA were performed in the protease-coupled assay using trypsin as isomer-specific protease. [b] The PPIase activities toward the substrates Suc-Ala-Glu-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Phe-pNA were measured using the protease-free assay.

Table 3: Characterization of the Interaction of **1** with Pin1 and Pin1 Variants[a]

| Pin1 | $\Delta H_{ITC}$ (kcal $mol^{-1}$) | $T\Delta S_{ITC}$ (kcal $mol^{-1}$) | $K_A$ ($10^7$ $M^{-1}$) | $1/K_I$[b] ($10^7$ $M^{-1}$) |
|---|---|---|---|---|
| wt | $-6.3 \pm 0.02$ | $4.1 \pm 0.01$ | $5.40 \pm 0.60$ | $4.9 \pm 1.0$ |
| Arg68Ala | $-6.4 \pm 0.03$ | $2.9 \pm 0.02$ | $0.77 \pm 0.25$ | $0.64 \pm 0.15$ |
| Arg69Ala | $-4.9 \pm 0.06$ | $3.1 \pm 0.03$ | $0.097 \pm 0.014$ | $0.095 \pm 0.006$ |
| Arg68/69Ala | nd[c] | nd | nd | $\ll 0.001$ |

[a] Conditions as described in Table 1. [b] Inhibition constants ($K_I$) were measured in the protease-free PPIase activity assay at 283 K using Suc-Ala-Glu-Pro-Phe-pNA as substrate. The results are shown as the means and SD from three independent determinations. [c] nd = not determined.

for binding. Measurements at lower pH values could not be performed since Pin1 tends to aggregate under these conditions.

*Kinetic and Thermodynamic Analysis of Pin1 Variants.* The two active site residues Arg68 and Arg69, which are thought to coordinate the phosphate group of the Pin1 substrate, are essential for efficient catalysis of the *cis/trans* isomerization of the Ser($PO_3H_2$)/Thr($PO_3H_2$)-Pro bond. To study the individual contributions of the single arginine side chains on binding and catalysis, we determined enzymatic efficiencies and the thermodynamic parameters for binding of different ligands for the Arg68Ala and Arg69Ala variants and the double variant Arg68/69Ala of Pin1 in comparison to wt Pin1.

Specificity constants $k_{cat}/K_M$ were determined using peptide derivatives as substrates, containing either Ser($PO_3H_2$), Glu, or Ala residues in the amino acid position preceding proline (Table 2). By using a substrate with the Ala residue proceeding Pro, the wt Pin1, the single Arg variants as well as the double Arg variant showed very low catalytic activity in the same order of magnitude attributed to the desolvation mechanism of catalysis, which accounts for only a small part of rate enhancement by Pin1 (*3*). The enzymatic activities of the Pin1 single and double variants toward substrates containing a Ser($PO_3H_2$) residue in the amino acid position preceding proline decreased in comparison to wt Pin1 (Table 2). While the Arg68Ala substitution leads to a 6-fold reduction of the PPIase activity toward the phosphorylated substrate, the Arg69Ala substitution reduced the activity 10-fold (Table 2). These values compare favorably with previously determined $k_{cat}/K_M$ values observed for the variants Pin1 Arg68Leu, Arg69Leu, and Arg68/69Leu (*23*). Interestingly, in the case of catalysis of the Glu-Pro bond isomerization, the substitution of Arg68 had almost no effect on PPIase activity whereas substitution of Arg69 resulted in a 6-fold reduction of the activity.

These data suggest that the two basic residues Arg68 and Arg69 coordinate the binding of the phosphorylated substrate, Arg69 being the more important of the two, as underlined by data obtained from inhibition of the enzymatic activities of wt Pin1 and variants by the phosphorylated ligand **1**.

Using a competition assay with Suc-Ala-Glu-Pro-Phe-pNA, compound **1** gave $1/K_I$ values of $(4.9 \pm 1.0) \times 10^7$ $M^{-1}$, $(0.64 \pm 0.15) \times 10^7$ $M^{-1}$, and $(0.095 \pm 0.006) \times 10^7$ $M^{-1}$ for wt Pin1, Arg68Ala, and Arg69Ala variants, respectively (Table 3). The double variant Arg68/69Ala, on the other hand, is insensitive to the inhibitor up to a concentration of 10 $\mu$M. The similarity of $1/K_I$ and $K_A$ values revealed weak affinity of Suc-Ala-Glu-Pro-Phe-pNA for the active site of Pin1 variants and an exclusive attack at the PPIase site by **1**.

Consistently, ITC experiments in which wt Pin1, Pin1-Arg68Ala, or Pin1Arg69Ala variants were titrated with **1** revealed $K_A$ values nearly identical to the $1/K_I$ values (Figure 6, Table 3). While for the Arg68Ala variant the 7-fold lowered binding affinity is mainly due to the reduced entropic contribution, the Pin1Arg69Ala variant additionally exposes a more positive value for $\Delta H_{ITC}$ so that the association for the inhibitor is 55-fold weaker than that of wt Pin1, confirming the larger contribution of Arg69 to the binding of phosphorylated ligands. The thermodynamic parameters of Pin1Arg68/69Ala could not be determined by ITC because of the low affinity of **1** with a dissociation constant above 10 $\mu$M.

To address the effect of the inversed stereo center at Cα of the phosphorylated D-Thr of **1** on its binding properties, ITC titration experiments and PPIase inhibition studies with wt Pin1 were performed using the all L-amino acid form of the compound (Ac-Phe-L-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$, (**2**)). The thermodynamic parameters for the binding of wt Pin1 to **1** and **2** are compared in Table 4.

The L-configuration of the Cα stereo center at the position preceding proline of the ligand resulted in a decreased affinity toward Pin1 and its variants, leading to a decreased $K_A$ value of $(0.17 \pm 0.02) \times 10^7$ $M^{-1}$ as well as an decreased $1/K_I$ value of $(0.19 \pm 0.01) \times 10^7$ $M^{-1}$ for the interaction of **2** and wt Pin1. The less favorable binding is caused by both the entropic as well as the enthalpic term. Pin1Arg68Ala exposed a $1/K_I$ value of $(0.11 \pm 0.01) \times 10^7$ $M^{-1}$, while Pin1Arg69Ala was inhibited by **2** with a $1/K_I$ of $(0.023 \pm 0.001) \times 10^7$ $M^{-1}$. These data show that the importance of Arg69 for binding of phosphorylated moieties is superior to

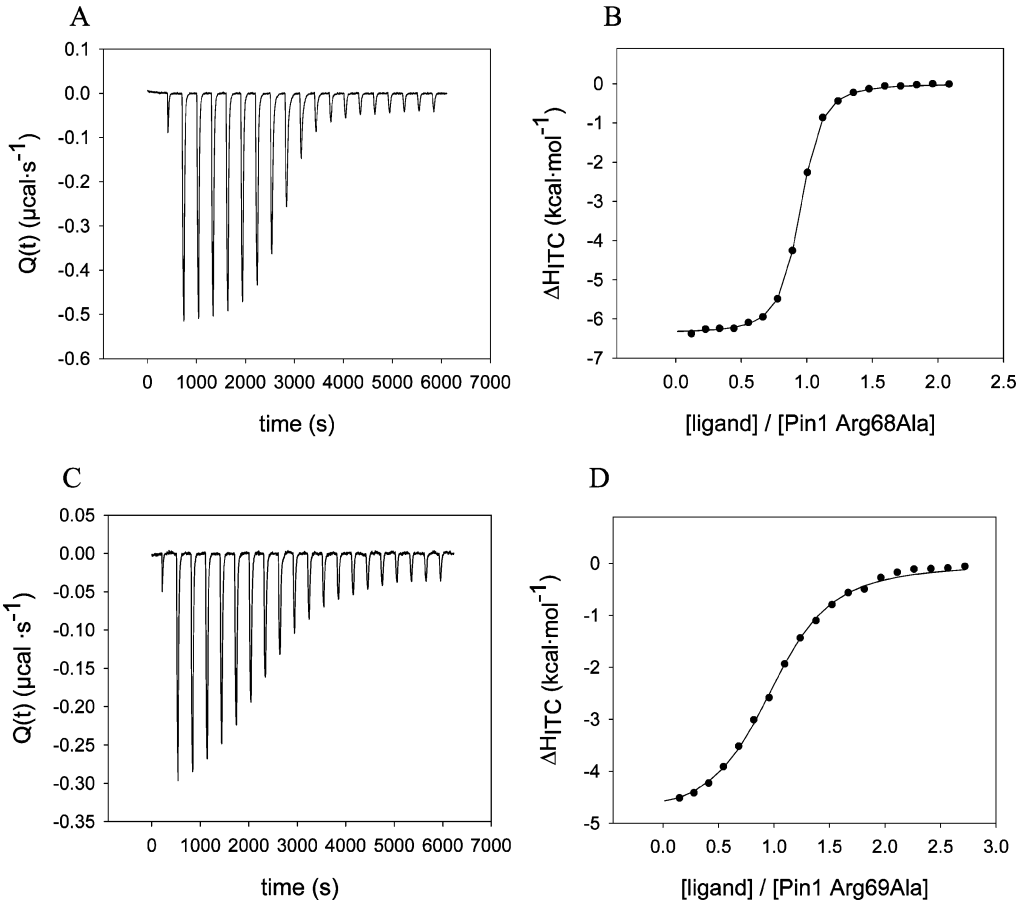


FIGURE 6: Calorimetric titration of Ac-Phe-D-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ (**1**) binding to Pin1 Arg68Ala (A, B) or Pin1 Arg69Ala (C, D). The titration was performed at 293 K in 35 mM HEPES buffer at pH 7.8. Prior to the experiment the instrument's sample cell was filled with 20 $\mu$M Arg68Ala or 13 $\mu$M Arg69Ala. Each peak (A, C) represents the injection of 15 $\mu$L of a 200 $\mu$M solution of **1** into the Pin1 variant solution. Integration of titration signals, caused by the interaction of Pin1 Arg68Ala or Pin1 Arg69Ala with **1**, resulted in $\Delta H_{ITC}$ values which were plotted against the molar ratio between ligand and the Pin1 variant (closed circles). The resulting titration curves (B, D) were fitted to a single binding site model by nonlinear least-squares analysis. The obtained thermodynamic parameters are depicted in Table 3.

Table 4: Comparison of the Binding Parameters of Diastereomeric Ligands **1** and **2** to wt Pin1[a]

| ligand | $\Delta H_{ITC}$ (kcal mol$^{-1}$) | $T\Delta S_{ITC}$ (kcal mol$^{-1}$) | $K_A$ ($10^7$ M$^{-1}$) | $1/K_I$[b] ($10^7$ M$^{-1}$) |
|---|---|---|---|---|
| **1** | $-6.3 \pm 0.02$ | $4.1 \pm 0.01$ | $5.40 \pm 0.60$ | $4.9 \pm 1.0$ |
| **2** | $-5.0 \pm 0.09$ | $3.3 \pm 0.20$ | $0.17 \pm 0.02$ | $0.19 \pm 0.01$ |

[a] Conditions as described in Table 1. [b] Inhibition constants ($K_I$) were measured in the protease-free PPIase activity assay at 283 K using Suc-Ala-Glu-Pro-Phe-pNA as substrate. The results are shown as the means and SD from three independent determinations.

Arg68 in both cases, applying the compound with the Cα stereo center of the phosphorylated Thr in D- or in L-configuration. Therefore we assume that the Cα configuration of the Thr($PO_3H_2$) residue does not influence the relative orientation of the two arginine side chains toward the phosphate moiety bound in the active site of Pin1. To further evaluate the importance of the phosphate group for the binding properties, the D-Thr($PO_3H_2$) group of **1** was replaced by D-Glu or L-Glu. For Ac-Phe-D-Glu-Pip-Nal-Gln-$NH_2$ (**3**) and Ac-Phe-L-Glu-Pip-Nal-Gln-$NH_2$ (**4**) $1/K_I$ values of $(1.49 \pm 0.42) \times 10^4$ M$^{-1}$ and $(0.69 \pm 0.06) \times 10^4$ M$^{-1}$ were determined, respectively. Incorporation of D-Glu results in a more potent inhibitor than incorporation of L-Glu. In all cases, glutamate poorly mimics the phosphothreonine in terms of Pin1 inhibition. The incorporation of L-Glu instead of L-Thr($PO_3H_2$) results in a 280-fold decreased of the association constant. This is in variance to the relation between L-Glu-Pro and L-Thr($PO_3H_2$)-Pro in Pin1 catalysis because glutamate mimics phospho-threonine quite well, resulting in a $k_{cat}/K_M$ value only 6.2-fold lower for Ala-Ala-L-Glu-Pro-Phe-pNA than Ala-Ala-L-Thr($PO_3H_2$)-Pro-Phe-pNA (*2*).

## DISCUSSION

The study presented here provides a comprehensive analysis of the thermodynamic parameters of the complex formation between the PPIase Pin1 and a substrate-derived ligand. Direct observation of the thermodynamics of Pin1 active site interactions with phosphorylated substrates is hampered by an additional phosphopeptide binding site in the WW domain of the protein and because the interaction between the active site of Pin1 and a particular peptide bond isomer is transient (*24*). In contrast, the recently developed substrate−analogue inhibitor Ac-Phe-D-Thr($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ (**1**) binds tightly to the PPIase domain in an exclusive manner. Consequently, when administered in HeLa cells by electroporation, **1** blocks cell proliferation in the G2/M phase at intracellular concentrations in the range of the cellular level of Pin1 (*28*). Its peptidic nature allows for the structural comparison of the inhibitor with substrates of Pin1. The introduction of a Pip residue in P1′ position

probably renders the peptide insensitive to the catalytic machinery of PPIases, as has already been shown for the example cyclophilin 18 (*36*). In addition, like other PPIases, Pin1 binds but does not catalytically isomerize peptides containing D-amino acids in P1 position (*24*, *37*). The formation of the adsorptive enzyme/inhibitory peptide complex of high stability thus might resemble transition-state binding of substrates. These properties allow for the first time the direct assessment of thermodynamic data for the interaction between the PPIase Pin1 and a phosphopeptide ligand. Complex formation is favored both enthalpically and entropically over the whole range of physiological temperatures; extrapolation of the linear dependence of $\Delta H_{ITC}$ and $T\Delta S_{ITC}$ on temperature shows that $T\Delta S_{ITC}$ will become unfavorable above 322 K, whereas $\Delta H_{ITC}$ will become unfavorable at temperatures below 252 K. By contrast, the formation of the complex between cyclophilin 18 and its tight binding inhibitor cyclosporine A is favored both enthalpically and entropically in a more narrow range of temperature. $T\Delta S_{ITC}$ was shown to become unfavorable above 289 K and $\Delta H_{ITC}$ was shown to become unfavorable below 264 K (*38*). The $\Delta G$ value remains almost unchanged over the temperature range due to enthalpy−entropy compensation, an effect discussed as an intrinsic property of perturbation of systems comprising multiple, weak, intermolecular forces such as protein/ligand interactions (*39*).

The negative change of the heat capacity $\Delta C_P$ calculated from the temperature dependence of $\Delta H_{ITC}$ is typical for ligand binding reactions. It correlates with the burial of surface area upon formation of the protein−ligand complexes (*40*). Binding of the ligand to Pin1 employs almost equal polar and apolar surface features. The amino acid side chains of the inhibitor are mostly hydrophobic, the phosphate group represents the major polar part. Therefore, electrostatic forces can be specifically assigned to interactions between the phosphoester group and positively charged residues of Pin1. They are likely responsible for the sensitivity of the association constants to elevated ionic strength with a decrease of the association constants $K_A$ to about 1.5% of the original value. Similarly, the catalytic efficiency of Pin1 decreases at increasing concentrations of NaCl, KCl, or inorganic phosphate (*3*, *25*). Since the catalytic machineries of cyclophilin 18 and FKBP12 tolerate NaCl at concentrations up to 2 M without a marked loss of efficacy, the decrease in affinity and catalytic efficiency at high ionic strength generated by 50−500 mM NaCl forms a special feature of Pin1. The lowered affinity of Pin1 toward Ac-Phe-D-Thr-($PO_3H_2$)-Pip-Nal-Gln-$NH_2$ in the presence of chloride ions is largely caused by more positive values of $\Delta H_{ITC}$ while the effect of $T\Delta S_{ITC}$ is small. The influence of inorganic phosphate on $\Delta H_{ITC}$ is by far stronger than that observed for chloride ions but is partially compensated by a more positive $T\Delta S_{ITC}$ term. Therefore we conclude that the decrease of affinity of Pin1 for **1** in the presence of the inorganic phosphate is caused by an additional specific interaction that adds to the effect of increased ionic strength. While chloride weakens protein−ligand interactions by shielding polyionic moieties of the protein, the inorganic oxyanions phosphate and sulfate are able to occupy the binding pocket by specific interaction with a basic cluster consisting of the triad Lys63, Arg68, and Arg69. Consequently, titration with **1** in the presence of inorganic phosphate lacks a large part of the energetic contribution provided by the interaction of the dianionic phosphoester group with the basic triad of Pin1. On the other hand, NMR spectroscopic investigations (*35*) demonstrated that the presence of a sulfate ion in the active site of Pin1 increases the rigidity of otherwise flexible side chains of Arg69, Trp73, and Ser114. Preformed rigidity due to the ion- and water-based hydrogen network in the presence of the inorganic oxyanion could explain the more favorable entropic effects for ligand binding in the presence of phosphate ions.

The physiological ionic strength is described to be $I = 0.15$ (*41*), and the cellular concentration of inorganic phosphate was recorded to be 2−5 mM (*35*). Under experimental conditions resembling the physiological environment, Pin1 revealed decreased catalytic efficiency toward its specific substrates of 1 order of magnitude, at least. The affinity toward **1** is reduced to a similar extent, indicating, that binding of **1** resembles transition state binding. It is obvious that the following question must be addressed: why does nature evolve an enzyme like Pin1 enabled with high catalytic power accompanied by features that permit the exhibition of only a minor part under physiological conditions? We hypothesize that the potential of bidentate binding of a substrate protein by Pin1 based on the existence of the two phosphopeptide binding domains improves the ability of the enzyme to target distinct substrates under certain cellular conditions. The existence of two phosphoester sites a certain distance from one another in a Pin1 substrate might reflect a means to increase the transition state affinity, thus compensating the reduction of affinity caused by the physiological ion composition of the cell selectively. However, experimental evidence for catalytic synergism when combining two phosphoester binding sites is still lacking. In contrast, two threonine phosphoester sites in a 40mer peptide derived from Tau protein separated by a segment of 19 amino acid residues resulted in a 3-fold lower *cis/trans* exchange rate at Thr($PO_3H_2$)212-Pro213 in an EXSY spectroscopy experiment when compared to the monophosphoester compound (*42*). This result draws attention to the distance between the phosphoester sites as a critical determinant of catalytic synergism. In this context it is interesting to note that endogenous proteins were often found to interact with Pin1 in their multiply phosphorylated forms, in the case of tau, the nuclear protein p54(nrb) Raf-1 (*43*−*45*). The hypothesis is consistent with the fact that the isolated PPIase domain of Pin1 (Pin1ΔWW) is sufficient to perform essential functions of Pin1 but only at high cellular concentrations (*6*). As a full length protein, Pin1 is an extremely efficient enzyme because less than 400 molecules per cell are required for yeast to survive under normal growth conditions (*46*).

The affinity of Pin1 to the inhibitor **1** expresses a strong pH dependency in the pH range between 5.5 and 7.0 and a minor decrease of affinity above pH 9.0. The simplest interpretation of these results includes ionizable amino acid side chain groups directly involved in active site binding of **1**, although indirect effects cannot be wholly excluded. The dissociation behavior at lower pH is characterized by a p$K_a$ value of 7.05 for the reactant state of the molecules, which is close to those of a phosphoester threonine side chain (*3*). In the Pin1/**1** complex, this p$K_a$ value is shifted to 5.74. This could be assigned to the preferential interaction of the phosphate group in the dianionic state with side chains of

the basic cluster Lys63/Arg68/Arg69 in Pin1 thus stabilizing phosphate as a dianion. At pH 5.5, the phosphate group of the unbound inhibitor is largely in the monoanionic state. Upon binding to Pin1, one proton will be released, yielding the dianionic state and coinciding with a proton transfer of $n = 0.77$. At pH 7.8, the phosphate group of the unbound inhibitor is largely in the dianionic state and no proton transfer takes place upon binding. The lack of a transferred proton at pH 7.8 suggests a simplified eq 7 with $\Delta H_{ITC} = \Delta H_{bind}$, where $\Delta H_{bind}$ is the sum of the binding enthalpies unaffected by protonation processes ($\Delta H'$) and the enthalpy change $\Delta H_{proton}$, a result of a given number ($n$) of protonation events (eq 8).

$$\Delta H_{bind} = \Delta H' + n\Delta H_{proton} \quad (8)$$

Equations 7 and 8 can be combined to form eq 9.

$$\Delta H_{ITC} = \Delta H' + n(\Delta H_{ion} + \Delta H_{proton}) \quad (9)$$

Presuming that at pH 7.8 no proton transfer takes place ($n = 0$), eq 9 can be converted to eq 10.

$$\Delta H_{proton(pH5.5)} = \frac{\Delta H_{ITC(pH5.5)} - \Delta H_{ITC(pH7.8)}}{n} - \Delta H_{ion} \quad (10)$$

Inserting the respective values for $\Delta H_{ITC}$ at pH 5.5 and 7.8, the appropriate buffer ionization enthalpy, and $n = 0.77$, eq 10 results in a value of 0.04 kcal mol$^{-1}$ as the average heat of deprotonation for one amino acid side chain. Phosphate ions have a very low heat of ionization (*47*). Accordingly, it can be assumed that the released proton can be allocated to the phosphate group of the inhibitor. However, the slope of the lower part of the experimental pH profile seems to deviate considerably from 1, suggesting a more complex dissociation behavior in this pH range. Two significant dissociation steps with similar p$K_A$ values of about 5.7 were already found by analyzing the pH dependency of the apparent $k_{cat}/K_M$ values of the Pin1-catalyzed isomerization of a phosphorylated peptide substrate (*3*). The phosphate group of the substrate and the imidazole side chain of the active site His59 were suggested to be the origin for these dissociation steps.

The second p$K_a$ value of 9.61 in the unbound state, shifting to a p$K_a \gg 9$ in the bound state, is not very well defined due to the lack of data at higher pH. It possibly reflects the dissociation of a cysteinyl, tyrosinyl, or lysyl side chain proton. One possible candidate in the proximity of the active site of Pin1 is Cys113. Although it is not directly involved in the catalytic mechanism of Pin1 (*48*), this amino acid residue seems to undergo a p$K_a$ shift upon ligand binding (*35*, *48*). The only other candidate in the proximity of the active site with an appropriate p$K_a$ is Lys63, which is part of the basic cluster involved in the interaction with oxyanions. However, it has to be mentioned that similar to prolyl bonds the phospho-D-Thr-Pip bond exists as *cis* and *trans* conformer. Considering that phospho-Thr-Pro bonds show a pH-dependent increase of the *cis* content with the two apparent p$K_a$ values 5.7 and 8.7 for the peptide Ala-Ala-Thr($PO_3H_2$)-Pro-Phe-pNA (*3*), isomer-specific binding of the inhibitor to Pin1 may result in a complex profile of pH-dependent affinity.

The basic triad in the Pin1 active site as the origin of the high specificity toward the phosphorylated Ser or Thr side chains was demonstrated by the 500-fold reduction of $k_{cat}/K_M$ for phosphorylated substrate relative to the wild-type enzyme, while the activity remained identical to wt Pin1 with the unphosphorylated substrate (*2*). The analysis of the association constants of the inhibitor **1** revealed that the reduction of affinity of the Arg68Ala variant is caused exclusively by a less favorable entropic contribution. Favorable entropic contributions to the free energy of ligand binding are often interpreted in terms of displacement of water molecules from the ligand binding site. Therefore, the guanidinium group of Arg68 might recruit a water molecule that is released into bulk water upon binding of **1** and which is not present in the Pin1 Arg68Ala variant. In the crystal structure of Pin1 with an empty PPIase site (PDB: 1F8A), a water molecule can be found in a distance of 2.6 Å to a guanidinium nitrogen of Arg68 (*5*). A direct ionic interaction of Arg69 with the phosphoester group of the substrate can be inferred from a less favorable enthalpic term for the formation of the Pin1Arg69Ala/**1** when compared to the wt Pin1 complex. This is a surprising result, since the crystal structure of Pin1 suggests an ionic interaction between the oxyanion and Arg68 rather than Arg69 because of the closer contact between Arg68 and sulfate.

The specificity constants ($k_{cat}/K_M$) of a phosphopeptide substrate catalyzed by wt Pin1, Pin1Arg68Ala, and Pin1Arg69Ala showed a rank order similar to those obtained for the complex formation of the same variants with **1**. The association constants exhibited a 7-fold decreased affinity for the Arg68Ala variant and a 52- to 55-fold decreased affinity in the case of the Arg69Ala variant when compared to wt Pin1. The correlation between $k_{cat}/K_M$ and $K_I$ does not hold for oligopeptides with a Glu residue at the P1 subsite because of the considerable loss of inhibitory potency of **3** and **4**. The data strongly support the conclusion that a specific interaction forces the Thr($PO_3H_2$)-Pip moiety into a transition state analogue conformation that cannot be adopted by the Glu-Pip residue.

Despite high efficacy, Pin1 catalysis does not function by synergistic group contributions to transition state affinity. The dissociation constant $K_{TX}$ of a substrate in the transition state can be calculated by dividing the rate constant for the uncatalyzed reaction, $k_{uncat}$, by the second-order rate constant $k_{cat}/K_M$ (*49*). The rate constant for the uncatalyzed isomerization of Thr($PO_3H_2$)-Pro moieties is about $1.7 \times 10^{-3}$ s$^{-1}$, the $k_{cat}/K_M$ for the Pin1-catalyzed isomerization of the substrate Ala-Ala-Thr($PO_3H_2$)-Pro-Phe-pNA is $1.37 \times 10^6$ M$^{-1}$ s$^{-1}$, resulting in a $K_{TX}$ value of substrate binding in the transition state of $1.2 \times 10^{-9}$ M. The constituent parts of a Pin1 substrate, the phosphoester group and a proline-containing oligopeptide segment can be tested separately for Pin1 binding. For the unphosphorylated substrate Ala-Ala-Thr-Pro-Phe-pNA, the value of $K_{TX}$ is $3.2 \times 10^{-6}$ M. The dissociation constant of inorganic phosphate from Pin1 is $2 \times 10^{-3}$ M (*35*). The sum of the negative free energies of binding of the unphosphorylated substrate and the missing phosphate falls only 0.93 kcal mol$^{-1}$ short of the negative free energy of binding of the phosphorylated substrate in the transition state. Interestingly, the apparent Michaelis constant ($K_M$) of Pin1 for the best oligopeptide substrate identified, Trp-Phe-Tyr-Ser($PO_3H_2$)-Pro-Phe-pNA, was $1 \times$

$10^{-5}$ M (*2*), whereas the dissociation constant $K_D$ for binding of the peptidic inhibitor **1** with $1.8 \times 10^{-8}$ M resembles more closely the dissociation constant $K_{TX}$ of a substrate in the transition state.

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