Role of Phosphorylation in Determining the Backbone Dynamics of the Serine/Threonine-Proline Motif and Pin1 Substrate Recognition†

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ABSTRACT: Proline residues provide a backbone switch in a polypeptide chain, which is controlled by the cis/trans isomerization about the peptidyl−prolyl bond. Phosphorylation of serine- and threonine-proline motifs has been shown to be a critical regulatory event for many proteins. The biological significance of these motifs has been further highlighted by the discovery of a novel and essential peptidyl−prolyl cis/trans isomerase Pin1. Pin1 is required for progression through mitosis via catalyzing the isomerization of phosphorylated Ser/Thr-Pro motifs specifically present in mitosis-specific phosphoproteins. However, little is known whether the phosphorylation regulates the conformational switch of the Ser/Thr-Pro bonds. Here, we report the synthesis and conformational characterization of a series of peptides that contain the phosphorylated or nonphosphorylated Ser/Thr-Pro motifs. Phosphorylation affected the rate of the cis to trans isomerization of the Thr/Ser-Pro bonds. As determined by a protease-coupled assay, the isomerization rate of phosphorylated Thr-Pro bond was found to be 8-fold slower than that of the nonphosphorylated analogue. Furthermore, studies of the pH dependence of the isomerization of the phosphopeptides reveal that both cis content and the rate constant of prolyl cis to trans isomerization are lower for the dianionic state of the phosphothreonine-containing peptides. These effects of phosphorylation are specific for phosphorylated Ser/Thr since neither phosphorylated Tyr nor glutamic acid was able to affect the prolyl isomerization. Finally, our experiments provide evidence that effective catalysis of cis/trans isomerization of phosphorylated Ser/Thr-Pro bonds by Pin1 is specific to the dianionic form of the substrate. Thus, our results demonstrate that protein phosphorylation specifically regulates the backbone dynamics of the Ser/Thr-Pro motifs and that Pin1 specifically isomerizes the certain conformation of the phosphorylated Ser/Thr-Pro motifs.

Protein phosphorylation and dephosphorylation on serine and threonine side chains is one of the most common post-translational protein modifications, which is catalyzed by protein Ser/Thr kinases (reviewed in refs *1* and *2*) and phosphatases (reviewed in refs *3*−*5*), respectively. Several models have been proposed to explain how phosphorylation of Ser/Thr side chains is able to regulate the function of a protein (*6*). It was deduced from crystallographic studies of glycogen phosphorylase in the phosphorylated (active) and the nonphosphorylated (inactive) forms that phosphorylation at Ser14 induces local conformational changes, resulting in the allosteric activation of the active site about 35 Å away by domain rotation (*7*−*10*). In contrast, phosphorylation of isocitrate dehydrogenase at Ser113 in the active site prevents the binding of the negatively charged substrate without additionally conformational changes of the enzyme (*11*−*13*). In the case of the cAMP-dependent protein kinase (PKA), phosphorylation at Thr197 is used to maintain the active site residues in their correct conformations (*14, 15*) and to control the affinity for its regulatory subunit (*16, 17*). In addition, phosphorylation-induced conformational changes are believed to be responsible for the observed increased thermal stability of protein kinase Cα (*18*) and the proto-oncogene product c-Fos as well as for the decreased sensitivity to intracellular proteolysis (*18*−*26*).

A number of protein kinases phosphorylate serine or threonine residue immediately preceding a proline residue in proteins, referred to as proline-directed protein kinases (*27*−*29*).

-Ser/Thr-Pro-Xaa-Lys/Arg	cdc2 kinase
Leu/Pro-Xaa-**Ser/Thr-Pro-**	MAP kinase

They include the well-known mitogen-activated protein (MAP)[1] kinases (reviewed in refs *28* and *30*) and cyclin-dependent protein kinases (Cdks), which play critical roles in signal transduction and in regulating the eukaryotic cell cycle, respectively. For example, at the G2/M transition, there is a rapid increase in Cdc2 kinase activity, leading to phosphorylation of a large number of proteins on Ser/Thr-

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Pro motifs (reviewed in refs *31* and *32*). It is believed that the phosphorylation of these proteins triggers many mitotic events such as reorganization of the microtubule network (*33*−*37*), condensation of chromosomes (*38*−*41*), and disassembly/assembly of the nuclear envelope and lamina (*42*, *43*).

Interestingly, proline residues introduce a backbone switch into a polypeptide chain by the cis/trans isomerization about the prolyl bond.[2] Phosphorylation of a protein on Ser/Thr-Pro motifs would be expected to generate an additional structural basis for the functional modification of proteins. This prediction has been recently substantiated by the discovery of the highly specialized peptidyl-prolyl cis/trans isomerase (PPIase) Pin1 (*44*). Pin1 is a conserved PPIase that is distinct from two other families of conventional PPIases, cyclophilins, and FKBPs, but is essential for mitosis. The crystal structure of Pin1 complexed with the dipeptide Ala-Pro suggests that Pin1 displays a unique substrate specificity (*54*). It prefers an acidic residue preceding proline due to interaction of the acidic side chain with a basic cluster in Pin1 consisting of the highly conserved residues Lys63, Arg68, and Arg69 at the entrance to the active site. Therefore, it was previously hypothesized that Pin1 may recognize its substrates in a phosphorylation-dependent manner (*54*). We have recently found that Pin1 binds and appears to regulate a highly conserved subset of proteins that undergo mitosis-specific phosphorylation (*45*). Furthermore, Pin1 specifically binds and effectively catalyzes the prolyl isomerization of phosphorylated Ser/Thr-Pro motifs that are present in mitosis-specific phosphoproteins (*46*). Therefore, Pin1 regulates cell cycle progression via a novel mechanism that is sequence-specific and phosphorylation-dependent prolyl isomerization (*46*). However, little is known about how phosphorylation affects the backbone dynamics of the Ser/Thr-Pro motif and Pin1 substrate recognition.

We report the synthesis and conformational characterization of chromogenic tetra- and pentapeptides. These oligopeptides not only are suitable for probing the backbone conformation but also contain putative functional groups thought to be important for intramolecular control of backbone conformation by side-chain phosphate esters. The cis to trans isomerization rate of Thr-Pro-containing peptides was found to be considerably slower upon phosphorylation. Analysis of the cis/trans isomerization of the side-chain phosphorylated peptides as a function of pH showed that the isomerization rate was much slower in the dianionic form of the peptides when compared with the monoprotonated form. Furthermore, it is this dianionic form of the Ser/Thr phosphate that was found to be a good substrate for Pin1. Thus, our results demonstrate that protein phosphorylation specifically regulates the backbone dynamics of the phosphorylated Ser/Thr-Pro motifs and that the dianionic form of Ser/Thr phosphate is likely more important for biological regulation.

EXPERIMENTAL SECTION

Peptide Synthesis. Peptides were synthesized either by global phosphorylation in solution or on resin bound peptides using dibenzyl-*N*,*N*-diisopropylphosphoramidite/tetrazole/MCPBA according to Bernhardt et al. (*47*) or by the incorporation of Fmoc-Ser/Thr[PO(OBzl)OH]-OH (Novabiochem, Bad Soden, Germany) into the sequence. After deprotection, the peptides were purified by RP-HPLC and lyophilized. Peptide identity was confirmed by ^{1}H, ^{13}C NMR, and ESI-MS. The purity of all peptides was checked by RP-HPLC and CE. Details of the syntheses are described in Supporting Information.

Enzymes. Bovine pancreas α-chymotrypsin was a product from Boehringer Mannheim (Germany), the content of active enzyme was determined by titration using 4-nitrophenyl acetate. Subtilisin Carlsberg (bacterial protease type VIII) was purchased from Sigma Chemical Co. (St. Louis); trypsin from bovine pancreas was a product from Serva (Heidelberg, Germany). N-Terminally His_6-tagged Pin1 was expressed, purified, and digested by thrombin according to Lu et al. (*44*). The GST-fused Pin1-wild-type and the mutant proteins Pin1(R68,69A) and Pin1(H59A) were expressed and purified according to Shen et al. (*45*). All measurements were performed by monitoring the absorbance of released 4-nitroaniline at 390 nm ($\epsilon = 11.814\ M^{-1}\ cm^{-1}$) on a Zeiss S 10 UV−vis spectralphotometer or on a Hewlett-Packard 8452A diode array spectrophotometer. Both spectralphotometers were equipped with a thermostated cuvette holder, and the temperature within the cell was constantly maintained by water circulated from a cryostat Haake D8 (Haake Fisons, Germany). The enzymatic kinetic data were analyzed using Aspect plus, version 1.1 (Carl Zeiss Jena GmbH, Germany), the Hewlett-Packard 89531A MS-DOS−UV−VIS operation software, and Sigma Plot Scientific Graphing System version 5.01 (Jandel Corp.).

Isomer-Specific Proteolysis. Comparison of the bimolecular rate constants k_{cat}/K_m for the chymotryptic (see Supporting Information) hydrolysis at pH 7.8 indicated that phosphopeptides are poorer substrates than their nonphosphorylated analogues. However, the determined k_{cat} values considerably exceed the expected rates of peptidyl-prolyl cis/trans isomerization and, thus, are sufficiently high to allow the very fast cleavage of the trans isomer in the protease coupled assay according to Fischer et al. (*48*). We routinely used α-chymotrypsin as an isomer-specific protease for substrates containing the Ala-Ala-Xaa-Pro-Phe-NH-Np sequence. The protease stock solution was prepared by dissolving 80 mg of α-chymotrypsin in 400 μL of 1 mM HCl and diluting the resulting mixture with 600 μL of buffer. The final concentration of chymotrypsin was between 1 and 14 mg/mL. In the case of substrates containing Ac-Ala-Xaa-Pro-Tyr-NH-Np, subtilisin Carlsberg was instead used for the assay at the final concentrations between 0.1 and 0.4

[1] Abbreviations: All amino acids are in the L configuration; Ac, acetyl; δ, chemical shift; CE, capillary electrophoresis; DIEA, *N*,*N*-diisopropylethylamine; DMAP, *N*, *N*-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; ESI-MS, electrospray mass spectroscopy; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; ISP, isomer-specific proteolysis; MAP kinases, mitogen-activated protein kinases; MBHA, 4-methylbenzhydrylamine; MCPBA, *m*-chloroperoxybenzoic acid; NH-Np, 4-nitroanilide; *N*-methylpyrrolidone; NMR, nuclear magnetic resonance; Pin1, a human peptidyl-prolyl cis/trans isomerase isolated as a protein interacting with the mitotic kinase NIMA; PPIase, peptidyl-prolyl cis/trans isomerase; RP-HPLC, reversed-phase high performance liquid chromatography; Suc, succinyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate.

[2] The term prolyl bond used throughout the paper indicates the peptide bond preceding proline in an amino acid sequence and prolyl isomerization indicates the cis/trans isomerization of the peptide bond preceding proline.

mg/mL. For substrates of Ac-Ala-Xaa-Pro-Lys-NH-Np, trypsin was used at the final concentrations between 0.5 and 2 mg/mL. In a typical experiment, x μL of buffer and y μL of protease stock solution ($x + y = 800$ μL) were preincubated for 5 min. After the solution had reached thermal equilibrium, the reaction was initiated by addition of 2 μL of the stock solution of the peptide derivative (10 mg/mL in DMSO). To estimate the cis content in aqueous solution, the peptide derivative was dissolved in assay buffer and the reaction was started by the addition of a concentrated protease stock solution.

Temperature Dependence of the Rate Constant $k_{cis\rightarrow trans}$. Assays were performed as described above for the ISP with the following exception. The temperature was varied between 10 and 30 °C in the case of the phosphopeptides and between 5.6 and 20 °C in the case of the nonphosphorylated peptides. The thermodynamic parameters of these interconversions were computed by linear regression analysis of the Eyring plot data.

Peptidyl-Prolyl cis/trans Isomerase Assays. The determination of the bimolecular rate constants k_{cat}/K_m for the PPIase catalyzed isomerization was performed by a protocol modified from Fischer et al. (*48*). Typically, a mixture of (1200-$x-y$) μL 35 mM Hepes, pH 7.8, and y μL (2−20 μL) trypsin stock solution (75 mg/mL) was treated with x μL PPIase solution reaching a final Pin1 concentration up to 80 nM. Reactions were started by addition of 1 μL of the peptide stock solutions (25 mg/mL) in DMSO. In the case of peptide substrates containing Leu/Phe-NH-Np, α-chymotrypsin stock solution (130 mg/mL) was used instead of trypsin. Since $[S]_o \ll K_m$ was valid, absorbance data points were fitted to a first-order kinetics. The k_{cat}/K_m values were calculated according to the equation $k_{cat}/K_m = (k_{obs} - k_u)/[PPIase]$ (*49*) with k_u = first-order rate constant for spontaneous cis/trans isomerization and k_{obs} = pseudo-first-order rate constant for cis/trans isomerization in the presence of PPIase. Determination of the pH dependence of k_{cat}/K_m was performed using a series of constant ionic strength buffers containing 25 mM sodium acetate, 0.1 M KCl ($5 \leq$ pH ≤ 6), and 25 mM Bis-Tris propane and 0.1 M KCl ($6 \leq$ pH ≤ 9.5). The final PPIase concentration was varied between 2 nM (pH 9.5) and 80 nM (pH 5). The reaction was initiated by addition of 1 μL of substrate solution. For ion strength inhibition assays, the indicated concentration of potassium chloride was added to 20 mM Tris/HCl buffer following adjustment to pH 7.8.

CD spectroscopy. CD data were collected on a Jasco J-710 spectropolarimeter (Tokyo, Japan) interfaced to a personal computer. Measurements were carried out in a thermostated quartz cell of the cylindrical 0.1 cm path length using aqueous solutions of the peptide derivatives at 10 °C. Baselines for all spectra were corrected by subtracting baselines for the appropriate solutions without peptide. Jasco software version 1,33.00 was used to calculate molar ellipticity based on the supplied sample molarity and cell path length. In general, 16 transients were obtained to improve the signal-to-noise ratio.

Determination of Microscopic pK_a Values Using NMR Spectroscopy. NMR measurements were carried out on a Bruker ARX 500 NMR spectrometer with proton resonance frequency at 500.13 MHz and ^{31}P resonance at 202 MHz, respectively. All spectra were recorded at 295 K. Temperature calibration was performed using a 866 thermometer (Keithley). Samples for pH-dependent measurements contained 5−7 mg of peptide dissolved in a H_2O/D_2O mixture. The resulting deuteration ratio of the solvent was 10%. ^{1}H spectra were referenced to internal TSP at 0 ppm. ^{31}P spectra were referenced to 85% phosphoric acid in spherical container immersed in the sample. The pH of the sample was changed by adding KOH or HCl. All pH values were measured with a combination glass electrode and not corrected for isotope effects. The results were fitted to a modified Henderson−Hasselbalch equation (*50*) proceeding from two different pK_a values.

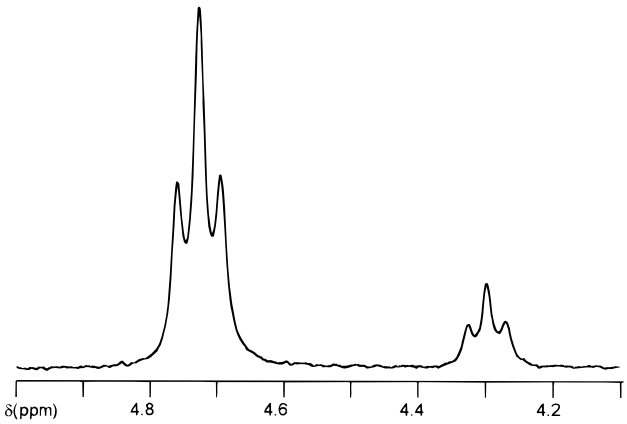

FIGURE 1: The ^{31}P NMR spectrum of a phosphoserine peptide. Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np was dissolved at a concentration of 14 mg/mL in H_2O/D_2O (10:1, v/v). The signals at 4.72 and 4.30 ppm represent the trans and cis isomer of the peptide derivative, respectively. We assigned the signal at 4.72 to the trans signal because of the percentage resulting from both isomer-specific proteolysis and the population of signals yielding the characteristic trans NOEs. The pH was adjusted to 8.2 using KOH. Phosphoric acid (85%) in a spherical container immersed in the sample was used as reference (0 ppm).

2D-NOESY (EXSY) experiments were acquired with the following parameters: spectral width of 5050 Hz in both dimensions, 16−32 transients, 512 time increments with 4048 complex points, and mixing times between 200 and 400 ms. Shifted sine bell squared weight functions were applied in both dimensions, and data were zero filled to a final size of 4K × 4 K. All resolved exchange peak/diagonal peak sets were used for integration and evaluation of prolyl isomerization rate constants (*51*). Determination of prolyl isomerization rate constants was done by the method described by Baine (*52*).

RESULTS

Microscopic pK_a Values of Ser/Thr Phosphates. To quantify the individual ionization of phosphorylated Ser/Thr-Pro-containing peptide-4-nitroanilides, pH titrations were carried out. We determined the pK_a values for the peptides Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np and Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np using ^{1}H and ^{31}P NMR spectroscopy. All ^{1}H and ^{31}P NMR spectra showed two sets of signals caused by cis/trans isomerization about the prolyl bond. We observed two signals in ^{31}P NMR spectra with different intensities but similar line shapes for each phosphopeptide (Figure 1).

The phosphorus resonance for the trans isomer of the peptide containing the phosphorylated Thr-Pro motif ap-

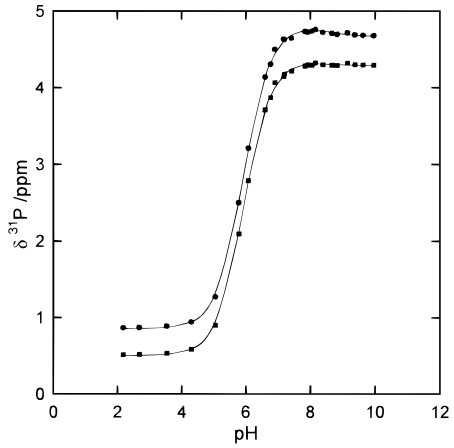

FIGURE 2: Isomer-specific ^{31}P chemical shifts as a function of pH. The ^{31}P chemical shift for the cis isomer (■) and the trans isomer (●) of Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np was plotted against the pH value. The pH was varied by adding KOH to the sample. Phosphoric acid (85%) in a spherical container immersed in the sample was used as reference (0 ppm). The solid lines represent the results of a fit to a modified Henderson−Hasselbalch equation (*50*) proceeding from two different pK_a values.

Table 1: Microscopic pK_a Values of Ala-Ala-Xaa(PO_3H_2)-Pro-Phe-NH-Np Determined by NMR Spectroscopy

-Xaa-	isomer	pK_{HPO_4}[a]	pK_{NH_3}[b]
-Thr-	cis	6.26	8.33
-Thr-	trans	5.95	8.56
-Ser-	cis	5.93	8.19
-Ser-	trans	5.92	8.48

[a] Obtained from ^{31}P NMR spectra. [b] Obtained from ^{1}H NMR spectra.

peared as a doublet with a ^{1}H-^{31}P *J* coupling constant of 9.0 Hz. The cis isomer caused a signal with an ^{1}H-^{31}P *J* coupling constant of 8.9 Hz. The intensity of the cis signal was 6.5% of the total intensity. In ^{31}P NMR spectra of the peptide containing the phosphorylated Ser-Pro motif, we found triplets with an ^{1}H-^{31}P J coupling constant of 6.5 and 5.7 Hz for the trans and the cis isomer, respectively. Variation of pH value between pH 2 and 10 led to a shift of the ^{31}P resonances by more than 3.8 ppm, as shown in Figure 2 for the peptide Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np. In ^{1}H NMR spectra pH-dependent shifts of 0.4 ppm for the β-methyl resonance of Ala[1] were observed for both isomers. The pK_{NH_3} values for the cis and trans isomers obtained by ^{31}P and ^{1}H NMR analyses according to a modified Henderson−Hasselbalch equation (50) were summarized in Table 1. The pK_a values for the PO_4^{2-} and HPO_4^{-} are similar to those reported by Hoffmann et al. (*53*) for phosphoserine or phosphothreonine-containing peptides. These results indicate that we are able to use the chemical-shift values from ^{1}H NMR spectra to determine isomer-specific pK_{NH_3} values. These values were later used to fit the ^{31}P NMR data obtained from two steps of protonation using the modified Henderson−Hasselbalch equation (*50*), as described below.

Influence of Phosphorylation on Isomerization of the Ser/Thr-Pro Motifs. Isomer-specific proteolysis (ISP) has been widely used to study the cis to trans isomerization of the peptidyl−prolyl bond (*48*). At very high concentrations of protease, the time course of the proteolytic cleavage of the phosphopeptides was of biphasic behavior (Figure 3). The slow phase was insensitive to the concentration of protease up to 400 mM. According to the conformational specificity

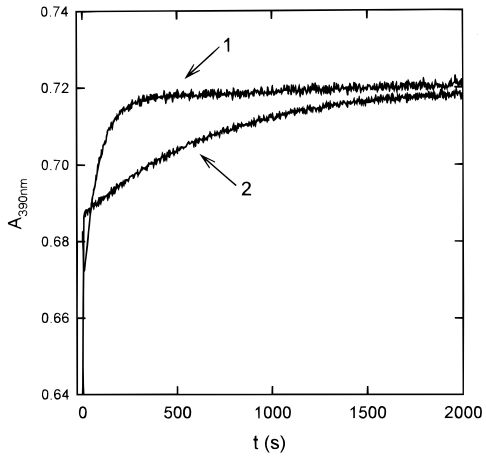

FIGURE 3: The time course of cis to trans isomerization of the prolyl bonds. Progress curves for the α-chymotrypsin mediated hydrolysis of Ac-Ala-Thr-Pro-Tyr-NH-Np (1) and Ac-Ala-Thr(PO_3H_2)-Pro-Tyr-NH-Np (2) visualized by isomer-specific production of 4-nitroaniline. The slow first-order traces are between 10 and 2000 s range. Data were collected at 10 °C in a pH 7.8 buffer containing 35 mM Hepes, as described in Experimental Procedures.

of a protease toward proline-containing peptides, the ratio of amplitudes of the phosphopeptides extrapolated to zero time represents the ratio of cis/trans isomers of the peptides in the stock solution. The percentage of the slow phases was the same as that of cis isomer found in the ^{1}H NMR spectra for all peptides investigated so far. For example, in the case of Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np, the ratio of the intensity was 6.4 in favor of the trans isomer as determined by analyzing the aromatic meta proton signal of the 4-nitroanilide residue (δ_{trans} = 8.22 ppm, δ_{cis} = 8.10 ppm). This value was almost identical to the ratio (6.3 ± 0.4) of two phases in the chymotrypsin-coupled assay. Thus, we were able to attribute the first-order rate constants representing the kinetics of the slow phase to the rate constant $k_{cis\rightarrow trans}$ of the phosphorylated Ser/Thr-Pro bond. Using the protease-coupled assay, we characterized the cis/trans isomerization of phosphorylated Ser/Thr-Pro bonds in a series of synthetic peptides, as compared with their corresponding nonphosphorylated analogues (Table 2). The prolyl isomerization of the phosphopeptides was slower compared to the corresponding nonphosphorylated compounds at 10 °C. This effect was more pronounced for peptides containing phosphothreonine preceding proline.

To examine whether these differences are specifically due to Ser/Thr phosphorylation, we first determined whether Tyr phosphorylation had a similar effect on isomerization of the Tyr-Pro bond. Interestingly, Ac-Ala-Tyr-Pro-Tyr-NH-Np and Ac-Ala-Tyr(PO_3H_2)-Pro-Tyr-NH-Np had similar isomerization kinetics (Table 2), indicating that phosphorylation of the Tyr-Pro motif is fairly insensitive to the cis/trans isomerization of the corresponding prolyl bond. Next, we examined the effect of free phosphothreonine on isomerization of a nonphosphorylated peptide. At concentrations even up to 1 mM, phosphothreonine did not affect prolyl bond isomerization of the model peptide derivative Suc-Ala-Ala-Pro-Leu-NH-Np, indicating that, in phosphorylated peptides, the effect must be due to intramolecular coupling of the phosphorylated Ser/Thr moiety to the rotating prolyl bond. Finally, we modified the side-chain phosphate moiety in the peptide. Significantly, esterification of the side-chain phos-

Table 2: Kinetic Constants of cis/trans Isomerization of Peptide-4-nitroanilides and Their Side-Chain Phosphorylated Analogues at pH 7.8

peptide derivative	cis content (%)[a,b]	$k_{cis\rightarrow trans} \times 10^3$ (s^{-1})[a,c]
Ala-Ala-Ser-Pro-Phe-NH-Np[d]	12.5 ± 0.2	9.7 ± 0.1
Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np[d]	17.5 ± 0.3	4.2 ± 0.2
Ala-Ala-Glu-Pro-Phe-NH-Np[d]	12.2 ± 0.1	6.9 ± 0.2
Ac-Ala-Ser-Pro-Lys-NH-Np[e]	10.7 ± 0.2	6.8 ± 0.1
Ac-Ala-Ser(PO_3H_2)-Pro-Lys-NH-Np[e]	12.4 ± 0.1	3.1 ± 0.1
Ac-Ala-Ser-Pro-Tyr-NH-Np[f]	13.4 ± 0.2	7.8 ± 0.5
Ac-Ala-Ser(PO_3H_2)-Pro-Tyr-NH-Np[f]	18.7 ± 0.3	4.0 ± 0.3
Ac-Ala-Ser(PO_3Me_2)-Pro-Tyr-NH-Np[f]	12.9 ± 0.7	15.2 ± 0.7
Ac-Ala-Ala-Ser-Pro-Arg-NH-Np[e]	8.8 ± 0.1	8.6 ± 0.4
Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np[e]	15.7 ± 0.2	3.3 ± 0.3
Ala-Ala-Thr-Pro-Phe-NH-Np[d]	10.2 ± 0.3	13.1 ± 0.8
Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np[d]	5.7 ± 0.4	1.7 ± 0.3
Ac-Ala-Thr-Pro-Lys-NH-Np[e]	8.5 ± 0.1	8.5 ± 0.3
Ac-Ala-Thr(PO_3H_2)-Pro-Lys-NH-Np[e]	6.4 ± 0.2	1.7 ± 0.2
Ac-Ala-Thr-Pro-Tyr-NH-Np[f]	13.2 ± 0.4	12.9 ± 0.5
Ac-Ala-Thr(PO_3H_2)-Pro-Tyr-NH-Np[f]	11.1 ± 0.3	1.8 ± 0.2
Ac-Ala-Ala-Thr-Pro-Arg-NH-Np[e]	8.9 ± 0.2	8.9 ± 0.2
Ac-Ala-Ala-Thr(PO_3H_2)-Pro-Arg-NH-Np[e]	10.3 ± 0.2	4.8 ± 0.1
Ac-Ala-Tyr-Pro-Tyr-NH-Np[f]	30.9 ± 0.6	5.1 ± 0.3
Ac-Ala-Tyr(PO_3H_2)-Pro-Tyr-NH-Np[f]	28.6 ± 0.8	3.9 ± 0.3

[a] The results are shown as the means and standard deviations from three independent determinations. [b] 35 mM Hepes. [c] 10 °C. [d] Measurements using chymotrypsin as isomer-specific protease. [e] Measurements using trypsin as isomer-specific protease. [f] Measurements using subtilisin as isomer-specific protease.

phate moiety prevented the phosphate from decelerating the prolyl bond isomerization, as shown in the peptide Ac-Ala-Ser(PO_3Me_2)-Pro-Tyr-NH-Np (Table 2). These results collectively demonstrate that Ser/Thr phosphorylation specifically hinders prolyl isomerization of the Ser/Thr-Pro bonds.

As depicted in Table 2, the thermodynamic equilibrium of Ser/Thr-Pro bond cis/trans isomerization is influenced by side-chain phosphorylation as well. The effect is most pronounced for peptides containing serine residues preceding proline because the cis content in aqueous solution increases from 8.8 to 15.7% in the phosphopeptide Ac-Ala-Ala-Ser-(PO_3H_2)-Pro-Arg-NH-Np at pH 7.8. However, influence of phosphorylation on the cis/trans ratio is still small in terms of the free energy differences $\Delta\Delta G$.

We determined the Eyring activation parameters for the cis/trans isomerization of the prolyl bond in peptide derivatives Ala-Ala-Xaa-Pro-Phe-NH-Np (Xaa = Ser/Thr, phoshoSer/Thr). These data are necessary in order to estimate whether the observed deceleration of prolyl bond isomerization (data obtained at 10 °C) by phosphorylation is relevant at physiological temperature. At 37 °C the rates of prolyl isomerization are to fast for reliable calculations. The thermodynamic constants obtained from linear Eyring plots in the temperature range of 10−30 °C are summarized in Table 3. The data assign the increase of rotational barrier of the phosphorylated Thr−Pro bond after deprotonation of the side-chain phosphate to an enthalpic contribution. This behavior permits extrapolations that indicate even greater rate differences caused by phosphorylation for physiological temperatures.

pH Dependence of Isomerization Constants. To further elucidate the role of Ser/Thr phosphorylation in controlling the conformation of phosphorylated Ser/Thr−Pro bond, we examined the effect of the side-chain phosphate protonation on the cis to trans prolyl isomerization by changing the assay

Table 3: Thermodynamic Constants of the Peptidyl-Prolyl cis/trans Isomerization of Ala-Ala-Xaa-Pro-Phe-NH-Np[a]

-Xaa-	pH	$\Delta S^{\#}$ (J mol^{-1} K^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ 25 °C (kJ mol^{-1})
-Ser-	7.8	4.1 ± 1.8	81.6 ± 0.2	80.4
-Ser(PO_3H_2)-	7.8	4.8 ± 11.2	83.5 ± 3.5	82.1
-Thr-	7.8	−11.2 ± 15.5	76.2 ± 4.4	79.5
-Thr(PO_3H_2)-	7.8	11.7 ± 4.4	87.6 ± 0.1	84.1
-Thr-	5.5	6.0 ± 4.0	81.2 ± 0.1	79.4
-Thr(PO_3H_2)-	5.5	−13.2 ± 16.1	78.2 ± 4.6	82.1

[a] Assays were performed using chymotrypsin as isomer-specific protease in a buffer containing 35 mM Hepes at the indicated pH.

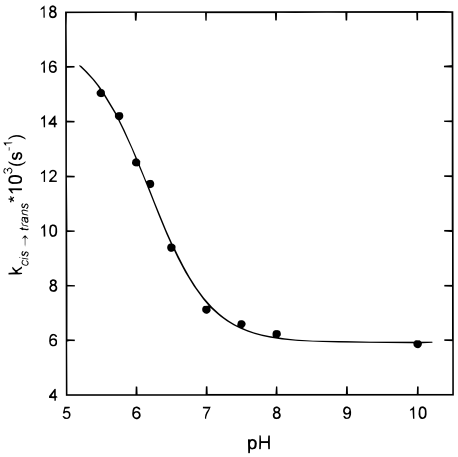

FIGURE 4: pH dependence of the cis to trans isomerization. pH dependence of the rate constant of the cis to trans isomerization of Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np was measured by isomer-specific proteolysis at 20 °C in buffers containing 25 mM sodium acetate 0.1 M KCl ($5.5 \leq pH \leq 6.5$) and 25 mM Bis-Tris propane 0.1 M KCl ($6.5 \leq pH \leq 10.0$). The solid line represents the best-fitting result based on the Henderson−Hasselbalch equation. The maximal cis content resulted from this fit is 18% at pH values below 4. The apparent microscopic pK_a value is 6.1 ± 0.1, which was almost the same as the estimated microscopic pK_a values of the side-chain phosphate moiety of the same peptide derivative (see Table 1).

pH. When four peptide derivatives Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np, Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np, Ac-Ala-Ala-Thr(PO_3H_2)-Pro-Arg-NH-Np and Ac-Ala-Ala-Ser-(PO_3H_2)-Pro-Arg-NH-Np, were subjected to ISP assay as a function of pH (from 5.5 to 10), the cis to trans isomerization rate increased after monoprotonation of the side-chain phosphate moiety. At pH 5.5, the rate constant $k_{cis\rightarrow trans}$ was about 3-fold higher compared to the value at pH 10 in the case of Ala-Ala-Thr(PO_3H_2)-Pro-Phe-NH-Np (Figure 4). Interestingly, pH did not have any detectable effects on the cis to trans isomerization of the peptides Ala-Ala-Glu-Pro-Phe-NH-Np and Ac-Ala-Ser(PO_3Me_2)-Pro-Tyr-NH-Np under the same assay conditions (data not shown). Furthermore, 2D NMR spectroscopic studies of Ala-Ala-Glu-Pro-Phe-NH-Np revealed that the introduction of a Glu residue preceding proline had no detectable effect on Glu-Pro bond cis/trans isomerization at two different pH conditions (pH 6.5 and 2.1). Since the pK_a value of the glutamic acid side chain is 4.4 as estimated by ^{1}H NMR spectroscopy, these results demonstrate that a simple negatively charged side chain cannot mimic the effect of the Ser/Thr side-chain phosphate.

The pH dependence of the cis content of Ala-Ala-Thr-(PO_3H_2)-Pro-Phe-NH-Np was evaluated between pH 2 and 10 using ^{1}H and ^{31}P NMR spectroscopy (Figure 5). The

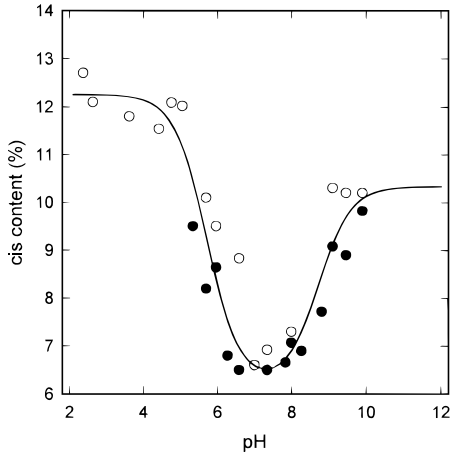

FIGURE 5: pH dependence of the content of the cis isomer of the phosphorylated Thr-Pro bond. The cis content of Ala-Ala-Thr-(PO_3H_2)-Pro-Phe-NH-Np was measured by ^{1}H (○) and ^{31}P (●) NMR spectroscopy in H_2O/D_2O (10:1, v/v). The pH was adjusted using KOH. Phosphoric acid (85%) in a spherical container immersed in the sample was used as reference (0 ppm) in the case of ^{31}P spectra. The solid line represents the best fitting results based on a modified Henderson−Hasselbalch equation (*50*) proceeding from two different pK_a values. Two apparent macroscopic pK_a values were 5.7 ± 0.2 and 8.7 ± 0.2, which are in good agreement with the isomer specific pK_a values estimated by NMR spectroscopy of the same peptide derivative (see Table 1).

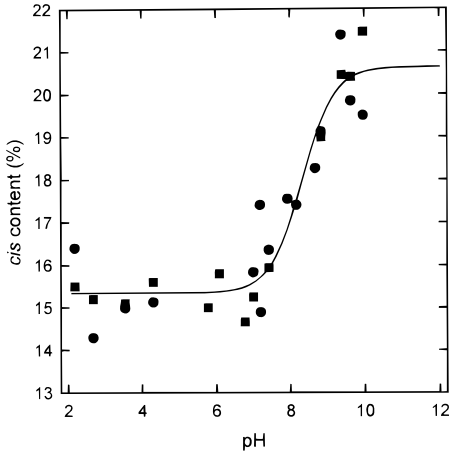

FIGURE 6: pH dependence of the content of the cis isomer of phosphorylated Ser-Pro. pH dependence of the cis content of Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np was measured by ^{1}H (■) and ^{31}P (●) NMR spectroscopy using the same procedure as described in Figure 5. The apparent macroscopic pK_a value of 8.3 ± 0.3 is very close to the microscopic pK_a values for the amino group of the same peptide derivative estimated by ^{1}H NMR spectroscopy (see Table 1).

pH/[cis] dependence indicated that the molecule has the N-terminus in a protonated state and the side chain in a dianionic state at pH 7. Fitting of the data to a modified Henderson−Hasselbalch equation proceeding from two different pK_a values yielded two apparent macroscopic pK_a values of 5.7 ± 0.2 and 8.7 ± 0.2, respectively. These values are in good agreement with the isomer specific pK_{HPO_4} and pK_{NH_3} values estimated by NMR spectroscopy of the same peptide derivative (see Table 1). These results suggest the cooperativity between the dissociation of the side-chain phosphate moiety (and N-terminal amino group) and the raised cis content.

Figure 6 shows the pH dependence of the cis content of Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np between pH 2 and 10 as measured by ^{1}H and ^{31}P NMR spectroscopy. Interestingly, despite the structural similarity between phosphothreonine and phosphoserine, the cis/trans ratio of phosphorylated Ser-Pro bond was not affected by dissociation of the side-chain phosphate moiety. The estimated apparent macroscopic pK_a value was 8.3 ± 0.3, suggesting that the deprotonation of the N-terminal amino group of Ala-Ala-Ser(PO_3H_2)-Pro-Phe-NH-Np (see Table 1 for isomer-specific pK_a values) is responsible for the change of the cis content from 15.3% to 20.6% when the pH of 6 is raised to 10. To rule out the possibility that a certain structure is formed in the phosphopeptides that could stabilize a certain isomeric state, we used UV−CD spectroscopy to compare the structures of the phosphoylated Ser/Thr peptides and their nonphosphorylated counterparts. When recorded in the range 190−260 nm in aqueous solution, all peptides so far examined had very similar spectra that are typical of extended chains (data not shown). Moreover, we were not able to detect any known secondary structure of the phosphopeptide-4-nitroanilides using ^{1}H NMR spectroscopy. Therefore, the above results demonstrate that the ionization of the phosphorylated side chain specifically affects the prolyl isomerization of the phosphorylated Ser/Thr-Pro peptides.

Dependence of Pin1-Catalyzed Prolyl Isomerization on pH. The PPIase Pin1 has a unique substrate specificity for phosphorylated Ser/Thr-Pro motifs, with k_{cat}/K_m values being increased up to 1300-fold after phosphorylation (*46*). We therefore next asked whether the ionization of the phosphorylated side chain of a serine residue preceding proline would affect the recognition of the substrate by Pin1. To address this question, we assayed Pin1 PPIase activity as a function of pH (from pH 5.0 to 9.5) at 10 °C. Dependence of k_{cat}/K_m on the pH value would be indicative for ionizable groups of free enzyme and free substrate required for approaching the transition state of the reaction. With regard to the amino acid residue preceding proline, three types of substrates were used: first, a substrate containing the phosphorylated Ser-Pro motif that represents, in principle, a bidendate anionic site; second, a substrate containing glutamic acid in this position that could potentially mimic a phosphate residue in its monoanionic state, although it is quite different from phosphate in nonenzymatic isomerization, as shown earlier; finally, a substrate equipped with the uncharged Ala-Pro bond (Figure 7).

The k_{cat}/K_m value for the Pin1-catalyzed isomerization of Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np showed a clear decrease in magnitude below pH 6.5 (Figure 7). It was impossible to fit the data to a model for a single significant dissociation step. Thus, we used a model for two kinetically significant dissociation steps. In the case of the phosphopeptide, two protons dissociate with similar pK_a values of about 5.7 ± 0.7 (Figure 7) for effective catalysis of prolyl isomerization by Pin1. It is obvious to hypothesize one kinetically significant single proton dissociation step for the substrate and the enzyme. A potential candidate protonated in this pH range is the imidazolic side chain of active-site His59 (*54*). We estimated the pH dependence of k_{cat}/K_m using the mutant PPIase GST-Pin1(H59A). This mutant protein shares the unique specificity of the wild-type but has a residual activity of 5.7% (*46*). We could not detect a dependence of k_{cat}/K_m on pH for this mutant protein if

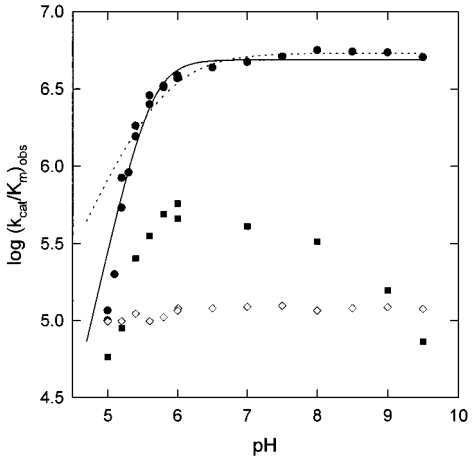

FIGURE 7: pH dependence of the Pin1-catalyzed cis/trans isomerization. pH dependence of the second-order rate constant k_{cat}/K_m for the Pin1-catalyzed cis/trans isomerization of Suc-Ala-Ala-Pro-Leu-NH-Np (□), Suc-Ala-Glu-Pro-Phe-NH-Np (■) and Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np (●). The enzymatic reaction was performed in buffers with a pH range 5−9.5. The buffers contain 25 mM sodium acetate 0.1 M KCl (5 ≤ pH ≤ 6) or 25 mM Bis-Tris propane 0.1 M KCl (6 ≤ pH ≤ 9.5) at 10 °C. The proteases trypsin and subtilisin were used for the ISP measurements of substrates containing Arg-NH-Np and Phe/Leu-NH-Np, respectively. The obtained data for Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np were fitted based on a reaction model that is either one or two kinetically significant proton dissociation steps represented by the broken and the solid line, respectively. The calculation produces similar pK_a values of 5.7 ± 0.7 for both dissociation steps.

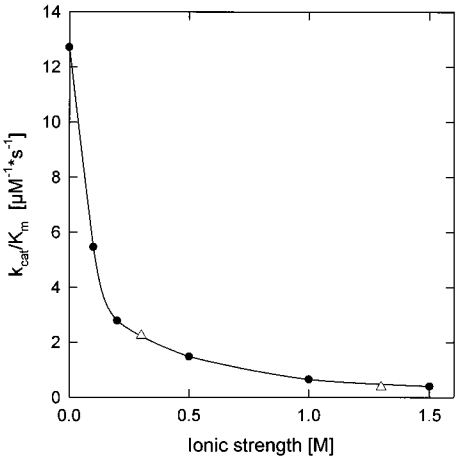

FIGURE 8: Inhibition of Pin1 PPIase activity by increasing ionic strength. The peptidyl-prolyl cis/trans isomerase activity of Pin1 was assayed at 10 °C using Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np as a substrate and trypsin as an isomer-specific protease. The buffer contained 20 mM Tris/HCl, pH 7.8, and the indicated concentrations of KCl (●) or NaCl (△). The solid line represents the best fitting result, with an apparent K_i value being 50 mM.

measured with Suc-Ala-Glu-Pro-Phe-NH-Np (data not shown). These results suggest that the side chain of His59 is likely responsible for the single dissociation step found for the Glu-Pro substrate below pH 6 and for one of the two protonation steps that can be calculated for the phosphorylated substrate. The decrease of the second-order rate constant for the Glu substrate at high pH values might indicate an involvement of a lysine residue in the enzyme mechanism. Indeed, a Lys side chain has been found to be located within the S_1 binding pocket[3] of Pin1 (*54*). The failure to detect an analogous pH dependence of k_{cat}/K_m for a substrate containing Ala−Pro bond (Figure 7) could be attributed to a catalysis by the overall hydrophobicity of the active site of Pin1. Probably the isomerization of all substrates which do not fulfill the Pin1 unique structural requirement of a negatively charged side chain preceding proline is catalyzed by the desolvatation mechanism, which contributes less than 10% to the PPIase mechanism of Pin1 (*2*).

Dependence of Pin1-Catalyzed Prolyl Isomerization on Ionic Strength. Inorganic phosphate has been previously shown to inhibit the catalysis of Glu−Pro bond isomerization by Pin1 and thereby proposed to have a specific affinity for the active site of Pin1 (*54*). In a related mechanism, ethylammonium salts are able to mimic the positively charged Lys side chain of a specific substrate for trypsin and mediate cleavage of unspecific substrate (*55*). Therefore, we examined whether inorganic phosphate alone could act in tandem with nonphosphorylated substrates to provide the specific substrate requirements for Pin1. When Pin1 PPIase activity was assayed using Suc-Ala-Ala-Pro-Leu-NH-Np or Ac-Ala-Ala-Ser-Pro-Arg-NH-Np as substrates in the presence of various concentrations of phosphate, we did not detect any synergistic effect between the nonphosphorylated substrates and inorganic phosphate up to 30 mM, but observed an inhibitory effect with higher concentrations of phosphate (data not shown). To determine whether this inhibitory effect is specific to the inorganic oxyanion or simply due to the altered ionic strength in the assay buffer, we investigated Pin1 activity toward a phosphopeptide substrate in buffers that were free of inorganic sulfate and phosphate, but contained increasing ionic strength by adding the monobasic chloride ion. We found a Pin1 activity dramatically decreased with increasing concentrations of salt (Figure 8). A 50% reduction of Pin1 activity was found at 50 mM KCl concentration. These results fail to provide any evidence for the specific affinity of inorganic phosphate to the active site of Pin1, as proposed previously (*54*). However, the strict dependence of Pin1 PPIase activity on ionic strength even in the case of a substrate without a negatively charged side chain preceding proline (Suc-Ala-Ala-Pro-Leu-NH-Np) again suggests that the electrostatic situation in the Pin1 active site is critical for a productive interaction between Pin1 and its substrate.

DISCUSSION

Prolyl isomerization is a slow interconversion in the polypeptide backbone that is frequently a rate-limiting step in refolding of proteins (*56*−*59*) and is thought to play a role in cellular restructuring of proteins. The relaxation times for unstructured peptide chains have been shown to be about 10 s at 37 °C (*60, 61*). In the cell, the rate-limiting prolyl isomerization can be avoided by catalysis through PPIases (*48, 59, 62, 63*). In many cellular events, the observed ratio of cis and trans isomers transiently does not correspond to the thermodynamic equilibrium under the actual conditions, but reflects the chemical environment of the peptide chain in an earlier state of the cellular situation. Therefore, there might be a lag phase in many physiological reactions that is sensitive to the spatial organization of polypeptide chains.

[3] The subsite nomenclature of Schechter and Berger is used to describe the positions relative to the scissile bond of the amino acid residues in the substrate [Schechter and Berger (1967) *Biochem. Biophys. Res. Commun. 27*, 157−162].

This lag may affect structure and function of a protein. In fact, it has been reported that cellular cyclophilin is able to accelerate the folding of procollagen I in suspended chick embryo tendon fibroblasts by a factor of only 1.7, and significantly, this small acceleration is sufficient to render procollagen to be overhydroxylated. This is because even a slightly prolonged resident time of the nontriple helical state cannot be tolerated in the endoplasmatic reticulum by the folding mechanism (*64*).

It has been shown that phosphorylation of Ser/Thr-Pro motifs causes changes in the tertiary structure of polypeptides. MAP or Cdc2 kinase mediated phosphorylation of Ser16 of chicken laminin B_2 induces disassembly of preformed head-to-tail polymers (*42, 65*−*68*). Hyperphosphorylation of Ser/Thr-Pro motifs of the microtuble-associated protein τ is responsible for the abnormal polymerization of tau yielding the so-called paired helical filaments (PHFs) (*25, 69*−*77*). These PHFs are structural components of neurofibrillary tangles. Together with the amyloid plaques, they are neuropathological hallmarks of Alzheimer's disease (*78, 79*). In addition, the major constituents of the cytoskeleton and filament proteins are regulated by phosphorylation of serine/threonine residues preceding proline, as shown in neurofilament proteins (*80*−*83*), smooth muscle caldesmon (*84*−*86*), skeletal muscle dystrophin (*87*), and synapsin I (*88*). In all these cases, structural changes and energetic changes of phosphoproteins have been discussed because phosphorylation changes the electrostatic state of proteins by introducing negative charges into the side chain of an amino acid residue. For the first time, we were able to provide a molecular description of the effects of phosphorylation on dynamics of the peptide backbone conformation. Phosphorylation of Ser/Thr-Pro motifs not only modulates both the thermodynamic equilibrium and the rate of cis/trans isomerization, but also regulates the specific interaction between phosphorylated Ser/Thr-Pro motifs and the unique PPIase Pin1.

It is well-known that the cis content markedly increases by the introduction of a negative charge (generation of a carboxylate group by deprotonation) in the case of acetylated N-alkyl amino acids and di- or tripeptides containing a C-terminal N-alkyl amino acid residue (*61, 89*−*91*). Our results show that phosphorylation of the Ser-Pro motif is accompanied by a change of the thermodynamic equilibrium of the prolyl bond isomerization at pH 7.8 (Table 2). These results indicate that the introduction of a negatively charged side chain affects the cis/trans ratio in a similar, although less pronounced, manner, as shown in peptides containing phosphoserine preceding proline. The significance of the negative charge at the phosphorylated serine side chain is further supported by the fact that similar cis contents are observed in the peptides Ac-Ala-Ser(PO_3Me_2)-Pro-Tyr-NH-Np and Ac-Ala-Ser-Pro-Tyr-NH-Np. However, this effect seems to be coupled to spatial localization of the negative charge since it is not observed for peptides containing Glu-Pro. Moreover, Tyr phosphorylation has no effect on phosphorylated Tyr-Pro bonds, ruling out the possibility that the observed effect is due to transmitting electrostatic phosphate group effects through covalent bonds to the rotating linkage (Table 2).

Interestingly, both isomerization and the isomeric ratio of a phosphorylated Ser/Thr-Pro segment are highly sensitive to slight pH variation in the physiological range. However, this is normally not the case for prolyl isomerization of a nonphosphorylated peptide bond, which is independent of pH in the range 5.0−9.0 as measured by ISP (*60, 92*) and between 1.8 and 10.8 as measured by ^{1}H NMR magnetization transfer (*93*). This unique feature of the prolyl bond is created by phosphorylation of Ser/Thr-Pro residues by proline-directed kinases within cells. Interestingly, although the phosphorylated Ser-Pro and Thr-Pro motifs are closely related regarding the side-chain structure and chemical properties, they have unexpected differences. The cis content of Ser-Pro-containing peptides becomes higher upon side-chain phosphorylation and is independent of the protonation state of the phosphate moiety. In contrast, in the case of the phosphorylated Thr-Pro motif, the cis content depends on the charge of the side chain, and the retardation of prolyl isomerization is also more pronounced. Although the exact underlying mechanism remains to be determined, these results may provide a basis for differential function and regulation of phosphorylated Ser-Pro and phosphorylated Thr-Pro sequences in proteins.

In addition, the introduction of a Glu/Asp residue for phosphorylated Ser/Thr residues have been frequently used to create constitutive phosphoSer/Thr mimic mutant proteins (*94*−*100*). However, this does not work in several cases (*99, 101*). We have now shown that Glu/Asp do not have the same properties as those of phosphorylated Ser/Thr, as shown by the pH dependence of the rate constant of prolyl isomerization and the alteration of the thermodynamic equilibrium. Therefore, the failure of Glu/Asp to substitute for phosphorylated Ser/Thr might be due to the differences in the local conformation of the peptide bonds.

In the proteins translated from open reading frames in the complete genomes of 6 microbial organisms, serine and threonine occur with a frequency between 4.0 and 9.0% (*102*−*107*). However, throughout the sequences, threonine covers a position preceding proline with higher propensity than that expected from its overall frequency. The Thr-Pro motif is found in repeating sequences typical of proteins that modify DNA structure (*108*) and plays an important role as a target for proline-directed phosphorylation (*109*). Only the His-Pro motif shows an analogous behavior. In parallel, the His−Pro bond exhibits an isomerization rate that is dependent on side-chain protonation (*110*). The higher propensity of threonine in a position preceding proline in connection with the sluggish prolyl isomerization toward phosphorylation of this motif might point to a special function that is possibly coupled to the unique dynamic behavior.

Finally, the alteration of the side-chain protonation state in a physiological pH range also controls the recognition of phosphorylated sequences by proteins, as shown by the interaction of Pin1 with the phosphorylated peptide derivative Ac-Ala-Ala-Ser(PO_3H_2)-Pro-Arg-NH-Np (Figure 7). Effective catalysis of isomerization of phosphorylated Ser/Thr−Pro bonds by Pin1 seems to be specific to the dianionic form of the substrate, and the unique specificity of Pin1 is only given in the context of a phosphate moiety that is covalently linked to a Ser/Thr-Pro motif. Therefore, phosphorylation not only affects the backbone dynamics of the Ser/Thr-Pro motifs, but also controls specific substrate recognition of the PPIases.

CONCLUSION

The effects of side-chain phosphorylation of Ser/Thr residues preceding proline on prolyl isomerization have been shown to be significant and diverse. In addition, to alter cis/trans equilibrium and the pH sensitivity of the introduced backbone switch, phosphorylation remarkably decreases the rate of peptidyl-prolyl cis/trans isomerization. These effects are not simply due to the negatively charged side chain since neither phosphotyrosine nor the widely used phosphoSer/Thr mimic glutamic acid is able to influence the prolyl isomerization in the same manner. Phosphorylation of Ser/Thr-Pro motifs is critical for biological recognition of the peptide bond by the extremely specialized PPIase Pin1. This modification generates a high-affinity module for Pin1, whose prolyl isomerase activity has been shown to be required for progression through mitosis of the cell cycle.

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SUPPORTING INFORMATION AVAILABLE

Detailed description of both phosphopeptide synthesis including ^{1}H and ^{13}C NMR data and characterization of side-chain phosphorylated peptide-4-nitroanilides as substrates for α-chymotrypsin are available from microfilm edition (11 pages). Ordering information is given on any current masthead page.

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