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Phosphorylation alters backbone conformational preferences of serine and threonine peptides

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ABSTRACT

Despite the notion that a control of protein function by phosphorylation works mainly by inducing its conformational changes, the phosphorylation effects on even small peptide conformation have not been fully understood yet. To study its possible effects on serine and threonine peptide conformations, we recently carried out pH- and temperature-dependent circular dichroism (CD) as well as ^{1}H NMR studies of the phosphorylated serine and threonine peptides and compared them with their unphosphorylated analogs. In the present article, by performing the self-consistent singular value decomposition analysis of the temperature-dependent CD spectra and by analyzing the $^3J(H_N,H_\alpha)$ coupling constants extracted from the NMR spectra, the populations of the polyproline II (PPII) and β-strand conformers of the phosphorylated Ser and Thr peptides are determined. As temperature is increased, the β-strand populations of both phosphorylated serine and threonine peptides increase. However, the dependences of PPII/β-strand population ratio on pH are different for these two cases. The phosphorylation of the serine peptide enhances the PPII propensity, whereas that of the threonine peptide has the opposite effect. This suggests that the serine and threonine phosphorylations can alter the backbone conformational propensity via direct but selective intramolecular hydrogen-bonding interactions with the peptide N—H groups. This clearly indicates that the phosphoryl group actively participates in modulating the peptide backbone conformations.

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Key words: peptide conformation; phosphorylation; polyproline II; blocked amino-acid; circular dichroism.

INTRODUCTION

Protein function and enzyme activity are often altered in several different ways. Particularly, a variety of post-translation modifications of proteins, such as acetylation, adenylation, methylation, and glycosylation to amino-acid side-chains, have been found to be of use to expand biological nature's inventory.[1] The most common type among them involves phosphorylation and dephosphorylation. Almost half of eukaryotic cell's proteins are known to be targets of phosphorylation under certain circumstances. Protein phosphorylation can induce a notable change of electrostatic environment in enzyme active site, modulate Coulombic interaction between proteins, or induce conformational changes that lead to alterations of a protein's globular structure and/or function.[2,3] Thereby, the protein phosphorylation plays an important role in a variety of catabolism/metabolism, signal transduction, gene expression, and ion channel.[4,5] Also, it has been shown that the activity of even small peptides can be regulated by phosphorylation. Therefore, it is immensely important to elucidate possible effects of the phosphorylation on the preferred backbone conformations of peptides.

The phosphate group is dianionic (negatively bivalent) at physiological conditions and forms strong electrostatic interactions with surrounding water molecules, neighboring peptides, and/or positively charged amino-acid side-chains.[2,3] To gain insights into the phosphorylation effect on the protein conformation, a few experimental and theoretical studies have been carried out within the context of short peptides and α-helices. Tholey *et al.*, for example, studied the phosphorylation effect on the backbone conformation of short tetrapeptides, Gly-Ser-Xaa-Ser, where Xaa is Ser, Thr, or Tyr in either phosphorylated or unphosphorylated form.[6] To predict the solution conformations of the GSSS and GSSpS tetrapeptides, Shen *et al.* carried out all-atom Brownian dynamics simulation studies.[7] Within the implicit solvent approximation, they concluded that both the GSSS and GSSpS conformations are close to an α-helix and the anionic

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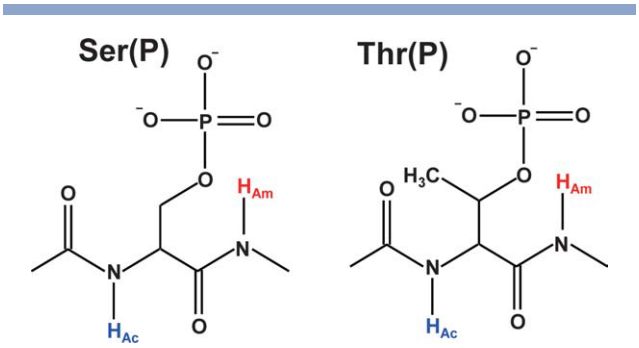

Figure 1

Molecular structures of phosphorylated serine and threonine peptides. The amino and carboxyl groups are blocked by acetylation and N-methyl amidation, respectively. The hydrogen atoms at the acetyl and amide ends are denoted as H_{Ac} and H_{Am}, respectively.

phosphate plays a role as a stabilizer for the α-helical conformation of GSSpS. Recently, Wong *et al.* performed both explicit TIP3P, SPC/E, and TIP4P and implicit GB/SA solvent molecular dynamics (MD) simulations for the same molecular systems by using the AMBER 7 program package with the *ff*99 force field parameters.[8] They studied the qualities of different solvent models and force field parameters as well as the effect of the phosphate charge state on intramolecular hydrogen-bonding propensity, and found that the ensemble-averaged properties are fairly insensitive to its charge state. Although they presented illustrating pictures on the strong intramolecular H-bonding propensity of the negatively charged phosphate, little information on the backbone conformations of GSSS and GSSpS was provided. We thus carried out FTIR, vibrational, and electronic circular dichroism (CD), and MD simulation studies of GSSS and GSSpS tetrapeptides and found that they adopt polyproline II (PPII) and β-strand conformations. In addition, the amide I IR band analysis of the GSSpS peptide provided direct evidence that the dianionic phosphate is capable of forming an intramolecular hydrogen bond with the N—H group of the phosphorylated serine residue.[9]

Recently, to extract detailed information on the intramolecular H-bonding interaction mechanism, we performed various spectroscopic studies of blocked serine and threonine peptides and their phosphorylated ones (Fig. 1). Note that these are the simplest possible model systems.[10] The amino and carboxyl ends of Ser and Thr are acetylated and amidated, respectively, to avoid any complexity induced by the interactions between the anionic phosphate and free amine and carboxyl groups in the cases of the phosphorylated serine and threonine peptides. Thus, each peptide has two different amide protons that are available for an intramolecular H-bond with the side-chain phosphate. We showed that both serine and threonine peptides can adopt two major conformations, that is, PPII and β-strand, and that they are predominant conformations even in the cases of their phosphorylated ones. Therefore, the general propensities for such conformations are not strongly affected by the side-chain β-methyl or phosphate group in these peptides. However, due to a lack of quantitative analysis method, we were not able to determine the percent populations of Ser(P) and Thr(P) peptide conformers in our previous work. Recently, we have developed the so-called self-consistent singular value decomposition (SC-SVD) analysis method to determine the relative populations of all possible trialanine conformers in water.[11,12] Here, we apply this SC-SVD protocol to the analyses of the temperature-dependent CD and NMR results of Ser(P) and Thr(P). It turns out that the phosphorylation of the serine residue at a higher pH (∼ 8.00) induces an increase of its PPII conformational preference, whereas that of the threonine residue lowers the population of PPII conformer. In contrast, there are little differences in such population distributions of PPII and β-strand at a lower pH ($<pK_{a2}$ of the phosphate group). These subtle but distinct differences between phosphorylated serine and threonine peptides in their intramolecular hydrogen-bonding interaction patterns could be pivotal in understanding the phosphorylation-induced conformational control of peptides and proteins.

EXPERIMENTAL METHODS

Most of the CD and NMR measurements were performed previously and reported in Ref. [10]. However, to complete the SC-SVD analyses of the temperature-dependent CD and NMR spectra, we needed finely controlled pH- and temperature-dependent CD and NMR spectra. Most importantly, the $^3J(H_N,H_\alpha)$ coupling constants of the Ser, Thr, Ser(P), and Thr(P) peptides had to be quantitatively measured for varying pH and temperature. Thus, we carried out such control experiments again. Hereafter, Ser, Thr, Ser(P), and Thr(P) denote the acetylated and amidated serine (Ac-Ser-NHMe), threonine (Ac-Thr-NHMe), phosphorylated serine (Ac-Ser(P)-NHMe), and phosphorylated threonine (Ac-Thr(P)-NHMe), respectively.

Circular dichroism

CD spectra were measured on Jasco J-810 CD spectrometer equipped with a Peltier-heating block. The sample concentration for all the CD measurements was 0.2 mg/mL, the sample cell used was 1.0 mm quartz, and the CD spectrometer sensitivity was adjusted to be 100 mdeg. The spectral resolution was 0.5 nm. The scanning mode and speed were continuous and 100 nm/min with 2 s response time, respectively. The frequency range was set to vary from 190 to 260 nm, and all the CD spectra presented here are averages taken over five independent scans. The solution temperature was increased from 0 to 80°C with a temperature interval to be 2°C.

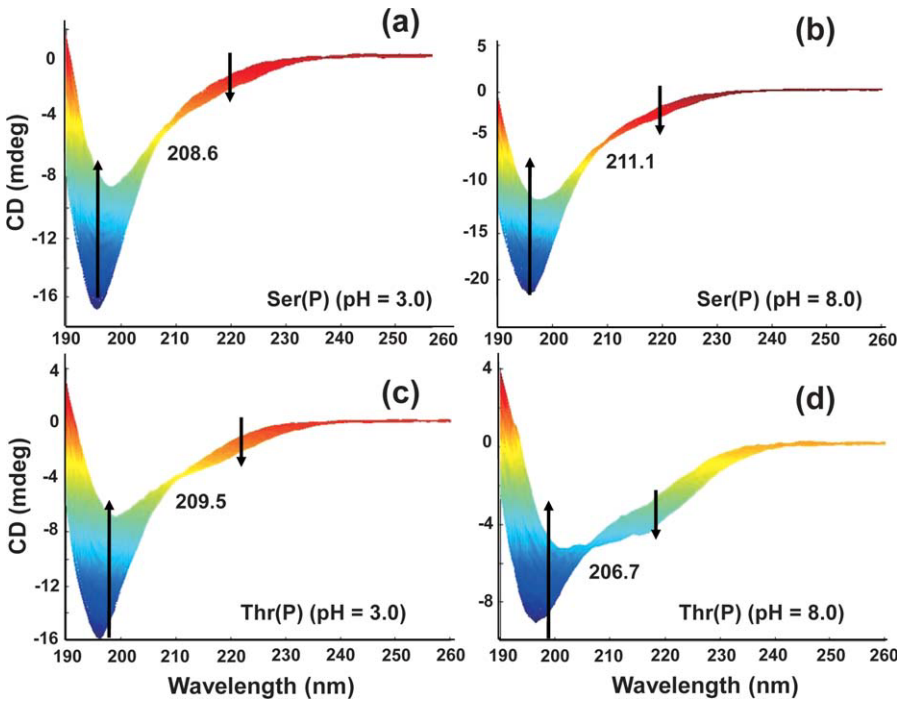

Figure 2

Temperature-dependent CD spectra of phosphorylated serine (**a** and **b**) and threonine (**c** and **d**) peptides at pH = 3.0 and 8.0. The wavelength of the isodichroic point is given in each figure.

NMR

The samples were dissolved in mixed H_2O/D_2O (= 9:1) solvent to a concentration of 5 mg/0.6 mL. Solution pH was adjusted by directly adding either 1*M* HCl or 1*M* NaOH solution to the sample solution. The pH range considered here is from 3.0 to 8.0. All the NMR spectra were measured on a Varian VnmrS 600 MHz NMR spectrometer using a triple resonance 5-mm HCN salt tolerant cold probe with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as a reference. Temperature was controlled by using nitrogen air cooler TC-84 (FTS Systems) and temperature controller L900 (Varian). ^{1}H NMR spectra were obtained using Noesypresat pulse sequence to suppress the water peak. For each sample, 64 transients were collected into 76,924 data points using a spectral width of 9615.4 Hz with a relaxation delay of 2.0 s and an acquisition time of 4.0 s. The data were analyzed with ACD Labs/NMR spectromanager to measure the chemical shifts of the two amide protons at the acetyl and amide ends and $^3J(H_N,H_\alpha)$ coupling constant. The original NMR free-induction-decay data were zero-filled to 524,288 points and then Fourier transformed. The resultant spectrum was fitted with a Gaussian + Lorentzian function. The peak-to-peak frequency that corresponds to the $^3J(H_N,H_\alpha)$ coupling constant was estimated by analyzing the fitted curves.

RESULTS

CD spectroscopy

Phosphorylated serine peptide

The T-dependent CD spectra of the Ser(P) at pH = 3.0 and 8.0 are shown in Figure 2(a,b), respectively. They both show a strong negative peak at 196 nm, which is a characteristic feature of typical PPII CD spectra[13–18], note that for the sake of completeness the T-dependent CD spectra of Ser and Thr at pH = 3.0 and 8.0 are also presented in Supporting Information. A broad negative band in the wavelength range from 210 to 230 nm is also observed, which originates from the contribution from the β-strand conformers. In addition, there is an isodichroic point at 208.6 (pH = 3.0) and 209.5 nm (pH = 8.0). This is particularly important observation revealing that there are two conformers, PPII and β-strand, and that the equilibrium shifts toward β-strand upon increasing temperature.[11,15] Such conformational change from PPII to β-strand, as temperature is increased, has been found in a number of short peptides including the unphosphorylated serine peptide (Ser) that is one of the reference model systems for this work. Therefore, it is believed that the general conformational preferences (PPII and β-strand) of serine peptide are not significantly influenced by the phosphorylation. However, as will be discussed below, the equilibrium con-

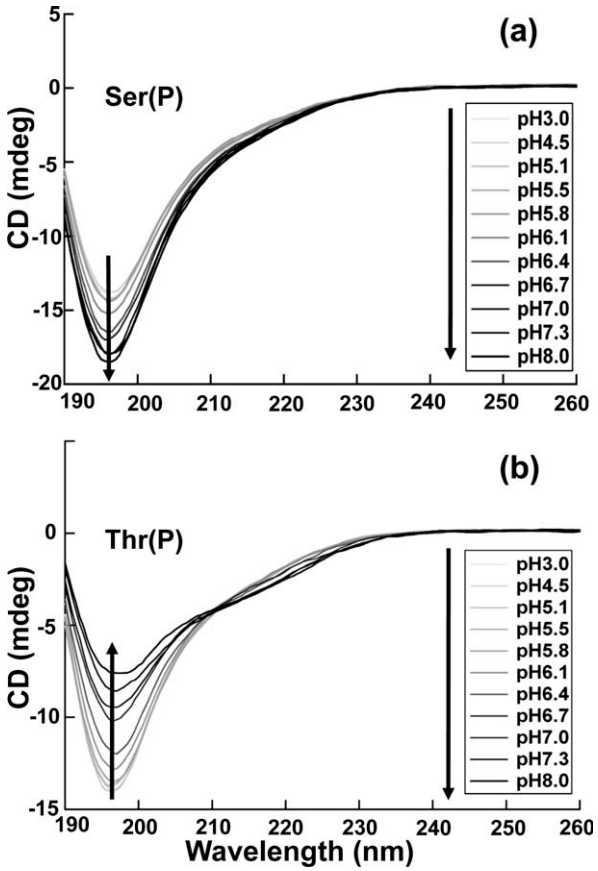

Figure 3

pH-dependent CD spectra of the phosphorylated serine (**a**) and threonine (**b**) peptides. As pH is increased, the negative peak intensity of Ser(P) at 195 nm increases. In contrast, the negative peak intensity of Thr(P) decreases, whereas the broad negative peak intensity at around 220 nm increases.

stant between PPII and β-strand conformers is affected by phosphorylation.

We next measured the CD spectra of Ser(P) and Thr(P) solutions for varying pH, to investigate the phosphate charge state effect on peptide backbone conformation (see Fig. 3). In the case of the Ser(P), the CD intensity of the negative peak at 196 nm increases as pH increases from 3.0 to 8.0, even though the overall spectral lineshape remains the same with no isodichroic points [see Fig. 3(a)]. This immediately suggests that the relative population of the PPII conformer increases, when the phosphate becomes dianionic at a higher pH (>pK_{a2}). The plot of the negative peak intensity at the wavelength of 196 nm versus pH, which can be found in Supporting Information, shows a typical sigmoidal shape and it was used to determine the pK_{a2} value of the phosphate group in Ser(P).

Phosphorylated threonine peptide

At a lower pH (= 3.0), the T-dependence of the Thr(P) CD is quantitatively similar to that of the Ser(P) [compare Fig. 2(a,c)]. Also, even at a higher pH (= 8.0), the temperature-dependent CD spectra of the Thr(P) exhibit a single isodichroic point at 206.7 nm, which again indicates a co-existence of the PPII and β-strand conformers. However, it is interesting to note that the negative peak intensity at around 220 nm is significantly stronger than that of the Ser(P) at the same pH (= 8.0) [compare Fig. 2(b,d)]. This means that the relative β-strand population of the Thr(P) at pH = 8.0 is significantly larger than that of the Ser(P) at this high pH. As a matter fact, such experimental observation was made before and discussed in Ref. [10]. This difference in the relative population of the β-strand conformer was attributed to the different intramolecular H-bonding patterns in the Ser(P) and the Thr(P). Unfortunately, we were not able to provide quantitative information on such population changes upon increasing temperature and pH previously in Ref. [10].

In the case of the Thr(P), another interesting observation was made while the pH-dependence of its CD spectrum was examined in detail [see Fig. 3(b)]. The presence of an isodichroic point at around 210 nm in Figure 3(b) shows that there is an equilibrium shift from PPII to β-strand conformers as the ionic state of the phosphate changes from monoanionic to dianionic forms. This is in stark contrast to the pH-dependence of the Ser(P) shown in Figure 3(a). To address this distinctive difference, we shall use experimentally estimated equilibrium constants and corresponding thermodynamic values including enthalpy and entropy differences between the PPII and β-strand conformers of all the phosphorylated and unphosphorylated serine and threonine peptides.

NMR spectroscopy

In Ref. [10], we reported only the NMR chemical shifts of the N—H protons in both the Ser(P) and Thr(P) for varying pH. With increased pH, the phosphate becomes dianionic at pH > 6. Thus, it can form an intramolecular H-bond with the nearby N—H hydrogen atoms. In the case of the Ser(P) (see Fig. 4), we found that the chemical shift of the acetyl-end peptide N—H proton, denoted as H_{Ac} in Figure 1, significantly changes at around pH = 5.88, which indicates that the pK_{a2} value of the Ser(P) phosphate in H_2O/D_2O (=9:1) solution is 5.88. However,

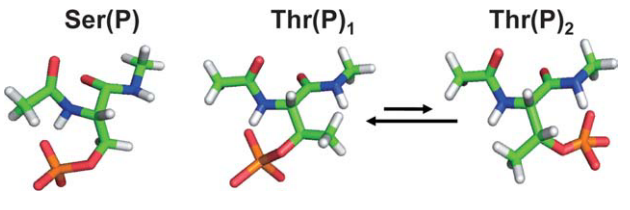

Figure 4

The phosphoryl group in Ser(P) at a higher pH exclusively interacts with the N—H group (H_{Ac}) of the serine. However, that in Thr(P) can form H-bond interactions with either H_{Ac} or H_{Am} atoms.

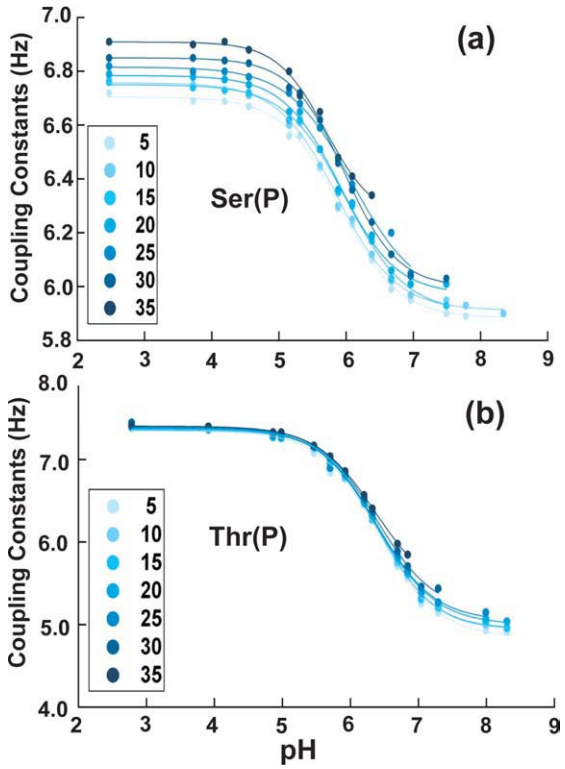

Figure 5

Temperature- and pH-dependent $^3J(H_N,H_\alpha)$ coupling constants of Ser(P) and Thr(P).

the chemical shift of the amide-end N—H proton, H_{Am}, remains constant irrespective of pH. This indicates that the amide-end N—H proton does not make any direct H-bonding interaction with neither the monoanionic nor dianionic phosphate. Here, it should be noted that the pK_{a1} value of the phosphate group is much smaller than 3.0.[19] Therefore, in the pH range from 3.0 to 8.0, the phosphate group is in a monoanionic or dianionic form.

In contrast, the chemical shifts of the two N—H protons, that is, H_{Ac} and H_{Am}, in the Thr(P) dissolved in H_2O/D_2O (= 9:1) increase as the phosphate becomes dianionic at pH > 6. The titration curve analysis showed that the pK_{a2} value of the phosphate in this threonine peptide in H_2O/D_2O(=9:1) solution is 6.33, which is slightly larger than that (5.88) of the phosphorylated serine peptide. These pK_{a2} values obtained from the titration curves of the N—H proton chemical shifts plotted with respect to pH are a bit different from those obtained by analyzing the pH-dependent CD spectra. Note that, in the latter case, the pK_{a2} values of the phosphorylated serine and threonine peptides were found to be 6.36 and 6.62, respectively (see Supporting Information). This can be understood by noting that the solvent used in the NMR measurements were H_2O/D_2O (= 9:1) mixed solvent, whereas that in the CD experiments was H_2O.

Another interesting observation is that the magnitude of the chemical shift change of the N—H_{Ac} proton is much larger than that of the N—H_{Am} proton in the case of the Thr(P). Perhaps, this indicates that the dianionic phosphate slightly prefers an H-bonding interaction with the N—H_{Ac} hydrogen atom instead of the amide-end N—H_{Am} atom in this case so that the equilibrium between $Thr(P)_1$ and $Thr(P)_2$ is shifted toward $Thr(P)_1$ (Fig. 4). Nonetheless, the pH-dependent NMR chemical shifts of the two N—H protons in the Thr(P) led us to believe that the phosphate can make intramolecular H-bonds with both N—H hydrogen atoms in this case of the dianionic Thr(P).

Next, we analyzed the $^3J(H_N,H_\alpha)$ coupling constants to extract quantitative information on the conformational changes induced by the intramolecular H-bonds in these two cases. Using the Karplus equation and the measured $^3J(H_N,H_\alpha)$ coupling constant, we can in principle determine the dihedral angle ϕ along the N—C_α bond. In Figure 5, the measured $^3J(H_N,H_\alpha)$ coupling constants for varying pH from 3 to 8 at different temperature in the range from 5 to 35°C are plotted. First of all, in the case of the Ser(P), the $^3J(H_N,H_\alpha)$ value at 10°C decreases from 6.77 to 5.90 Hz upon increasing pH. Noting that the $^3J(H_N,H_\alpha)$ value of PPII is typically smaller than that of β-strand, we could immediately conclude that the relative population of PPII increases when the Ser(P) phosphate becomes dianionic and forms an intramolecular H-bond with the N—H_{Ac} hydrogen atom. The pH-dependence of the $^3J(H_N,H_\alpha)$ coupling constant of the Thr(P) is essentially identical to that of the serine peptide. As pH is increased from 2.78 to 8.30, the coupling constant of the H_{Ac}—N—C_α—H group decreases from 7.39 to 4.97 Hz. Based on this observation, one may expect that the additional intramolecular H-bond formed between the dianionic phosphate and the amide-end N—H_{Am} group increases the PPII population.

However, as will be shown later in this paper, such qualitative interpretations solely based on the pH-dependence of the $^3J(H_N,H_\alpha)$ coupling constant could be erroneous. More specifically, we shall show that, even though the dianionic phosphate does enhance the PPII propensity of the serine peptide, the dianionic phosphate in the threonine peptide makes the population of β-strand conformer increase instead of PPII conformer. As discussed in our previous article,[10] it is the methyl group on the β-carbon atom in the Thr(P) that plays an important role in making such subtle differences in phosphorylation effects on serine and threonine.

ANALYSES AND DISCUSSION

Self-consistent SVD analysis method

The temperature-dependent NMR and CD results presented in the previous section reveal that both phosphorylated and unphosphorylated serine and threonine peptides adopt PPII and β-strand conformations. Now, to determine

the relative PPII and β-strand populations, we use the following procedure. The coupling constant $^3J(H_N,H_\alpha)$, denoted as J_{exp}, experimentally measured for a given N—H proton coupled to its neighboring C_α—H proton is assumed to be expressed as a linear combination of the reference coupling constants of the two conformers as,

$$J_{exp} = P_{PPII}J_{PPII} + P_\beta J_\beta = P_{PPII}J_{PPII} + (1 - P_{PPII})J_\beta, \quad (1)$$

where the relative population of the PPII (β-strand) conformer is denoted as P_{PPII} (P_β), and $P_{PPII} + P_\beta = 1$. The T-dependence of J_{exp} originates from the change of the relative populations of PPII and β-strand conformers for varying temperature. Here, J_{PPII} and J_β are the coupling constants when the corresponding peptide conformations are purely PPII and β-strand, respectively. These reference-coupling constants are assumed independent on temperature. In fact, these two values cannot be directly measured because the peptides have conformational distribution of the two conformers in general so that we shall treat them as adjustable parameters in this work. Thus, in Eq. (1), there are three unknowns, that is, P_{PPII}, J_{PPII}, and J_β. The temperature-dependent data on J_{exp} are not sufficient to determine these values unambiguously. Thus, we use the T-dependent CD spectra as an independent set of experimental data.

The experimentally measured CD spectrum $\Delta A_{exp}(\lambda)$ is similarly given as a weighted sum of the eigenspectra of PPII and β-strand conformers, that is,

$$\begin{aligned}\Delta A_{exp}(\lambda) &= P_{PPII}\Delta A_{PPII}(\lambda) + P_\beta \Delta A_\beta(\lambda) \\ &= P_{PPII}\Delta A_{PPII}(\lambda) + (1 - P_{PPII})\Delta A_\beta(\lambda). \quad (2)\end{aligned}$$

Here, the reference spectra, which are referred to as the eigenspectra of the involved conformers, PPII and β-strand, are denoted as $\Delta A_{PPII}(\lambda)$ and $\Delta A_\beta(\lambda)$, respectively. Much like the reference NMR coupling constants, J_{PPII} and J_β, one cannot experimentally measure $\Delta A_{PPII}(\lambda)$ and $\Delta A_\beta(\lambda)$ independently. Therefore, a singular value decomposition (SVD) method is used to determine these eigenspectra.[11] For a given peptide solution with concentration of c, the mathematical SVD analysis of $\Delta A_{exp}(\lambda, T)$ converts the matrix form of concentration-normalized spectrum into a sum of products of eigenspectrum, singular value, and purely T-dependent function as

$$\begin{aligned}\overline{\Delta A}_{exp}(\lambda, T) &\equiv \Delta A_{exp}(\lambda, T)/c = [\Delta A_1(\lambda) \\ &\Delta A_2(\lambda)]\begin{bmatrix} C_1 & 0 \\ 0 & C_2 \end{bmatrix}\begin{bmatrix} f_1(T) \\ f_2(T) \end{bmatrix} \\ &= \Delta A_1(\lambda)C_1 f_1(T) + \Delta A_2(\lambda)C_2 f_2(T), \quad (3)\end{aligned}$$

where $\Delta A_1(\lambda)$ and $\Delta A_2(\lambda)$ are the SVD-eigenspectra of the two dominant components, respectively. Actually, the singular value matrix $\tilde{C}$ obtained from this standard SVD analysis has N different diagonal elements with N being the number of experimentally varied temperatures in this case. However, for a two-species (two-conformer) system the first two singular values C_1 and C_2 are significantly larger than the remaining diagonal elements. Here, the two singular values represent the relative weights of the contributions from the two components to each CD spectrum. In Eq. (3), the T-dependent functions $f(T)$ provide information on the relative populations of the two components with respect to temperature.

However, one cannot directly interpret the values $C_1 f_1(T)$ and $C_2 f_2(T)$ at a given temperature T as the mole fractions of the two species at all. This is because the eigenspectra $\Delta A_1(\lambda)$ and $\Delta A_2(\lambda)$ are not the molar CD spectra of the two conformers but just mathematically determined eigenspectra of the first two dominant singular value components resulted from the standard SVD analysis. Thus, there should be a proper set of criteria to obtain molar eigenspectra and relative populations (or concentrations) of the two conformers. In this regard, one of the most important observations is the fact that there is an isodichroic point in the T-dependent CD spectra. We found that the raw eigenspectra $\Delta A_1(\lambda)$ and $\Delta A_2(\lambda)$ directly obtained from the above SVD analysis usually do not meet this isodichroic-point-criterion. Thus, we found it necessary to modify the SVD procedure. As shown recently by us, this can be achieved by using the following 2 by 2 identity matrix,

$$\begin{aligned}\begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} &= \begin{bmatrix} \cos\vartheta & \sin\vartheta \\ -\sin\vartheta & \cos\vartheta \end{bmatrix}\begin{bmatrix} \cos\vartheta & -\sin\vartheta \\ \sin\vartheta & \cos\vartheta \end{bmatrix} \\ &= U^{-1}(\vartheta)U(\vartheta). \quad (4)\end{aligned}$$

Inserting this identity matrix into Eq. (3), we can rewrite the equation as

$$\begin{aligned}\overline{\Delta A}_{exp}(\lambda, T) = [\Delta A_1(\lambda) \quad \Delta A_2(\lambda)]U^{-1}(\vartheta)U(\vartheta) \\ \begin{bmatrix} C_1 & 0 \\ 0 & C_2 \end{bmatrix}\begin{bmatrix} f_1(T) \\ f_2(T) \end{bmatrix}. \quad (5)\end{aligned}$$

Note that the rotation angle ϑ is considered another variational parameter in this case. Then, properly adjusting ϑ, we could obtain the transformed eigenspectra satisfying the isodichroic-point-criterion. Thus, we have

$$\Delta A_{exp}(\lambda, T) = \Delta A_{PPII}(\lambda)f_{PPII}(T) + \Delta A_\beta(\lambda)f_\beta(T), \quad (6)$$

where

$$\begin{aligned}\Delta A_{PPII}(\lambda) &= \Delta A_1(\lambda)\cos\vartheta - \Delta A_2(\lambda)\sin\vartheta \\ \Delta A_\beta(\lambda) &= \Delta A_1(\lambda)\sin\vartheta + \Delta A_2(\lambda)\cos\vartheta \\ f_{PPII}(T) &= C_1 f_1(T)\cos\vartheta - C_2 f_2(T)\sin\vartheta \\ f_\beta(T) &= C_1 f_1(T)\sin\vartheta + C_2 f_2(T)\cos\vartheta. \quad (7)\end{aligned}$$

Note that $\Delta A_{PPII}(\lambda) = \Delta A_\beta(\lambda)$ at the wavelength λ_{id} of the isodichroic point. However, still one cannot

Table I
Reference Coupling Constants of the PPII and β-Strand Conformers

	J_{PPII} (ϕ)	J_{β} (ϕ)
Ser(P) (pH = 3.0)	6.35 (−77°)	8.27 (−148°)
Ser(P) (pH = 8.0)	5.62 (−72°)	7.63 (−154°)
Thr(P) (pH = 3.0)	7.27 (−83°)	7.90 (−152°)
Thr(P) (pH = 8.0)	4.29 (−62°)	6.51 (−162°)
Ser	6.65 (−79°)	8.22 (−149°)
Thr	7.37 (−86°)	8.61 (−145°)

The dihedral ϕ-angles were calculated using these reference coupling constants and the Karplus equation with parameters in Ref. [31]. The calculated dihedral angles are given in parentheses. Although we controlled the pH values of the Ser(P) and Thr(P) solutions, we found that the Ser and Thr CD spectra do not depend on pH. The pH values of the Ser and Thr solutions used in the NMR measurements were 5.60 and 4.78. For the self-consistent singular value decomposition analyses, we used the T-dependent CD spectra of the Ser and Thr solutions at pH = 8.0 even though we also measured the T-dependent CD spectra of these solutions at pH = 3.0.

conclude that the above $\Delta A_{PPII}(\lambda)$ and $\Delta A_{\beta}(\lambda)$ are the molar CD spectra of the PPII and β-strand conformers yet, because the resulting $f_{PPII}(T)$ and $f_{\beta}(T)$ are not relative molar concentrations of the two conformers. Therefore, we need to introduce yet another parameter called the molar ellipticity ratio, $R_{PPII/\beta}$, which is the measure of molar CD intensities of the two conformers. With this additional variational parameter, Eq. (6) is rewritten as

$$\begin{aligned}\Delta A_{exp}(\lambda,T) &= \frac{R_{PPII/\beta}}{1+R_{PPII/\beta}}\Delta A_{PPII}(\lambda)f_{PPII}(T)\\ &+\frac{1}{1+R_{PPII/\beta}}\Delta A_{\beta}(\lambda)f_{\beta}(T)\\ &=\Delta\tilde{A}_{PPII}(\lambda)P_{PPII}(T)+\Delta\tilde{A}_{\beta}(\lambda)P_{\beta}(T). \qquad (8)\end{aligned}$$

Due to the newly multiplied constants $R_{PPII/\beta}/(1 + R_{PPII/\beta})$ and $1 / (1 + R_{PPII/\beta})$ to the first and second terms on the right-hand side of Eq. (6), the two eigenspectra $\Delta\tilde{A}_{PII}(\lambda)$ and $\Delta\tilde{A}_{\beta}(\lambda)$ defined in Eq. (8) do not satisfy the isodichroic-point-criterion, that is, $\Delta\tilde{A}_{PII}(\lambda_{id}) \neq \Delta\tilde{A}_{\beta}(\lambda_{id})$ at the wavelength λ_{id} of the isodichroic point. This problem can be solved in an iterative manner as follows. For an initially guessed $R_{PPII/\beta}$ value, we first determine the two eigenspectra $\Delta\tilde{A}_{PII}(\lambda)$ and $\Delta\tilde{A}_{\beta}(\lambda)$ that satisfy the isodichroic-point-criterion. This gives us the relative populations P_{PPII} and P_{β}. Then, the T-dependent equilibrium constants ($K_{eq}(T) = P_{\beta}(T)/P_{PPII}(T)$) are analyzed with the Van't Hoff equation. If the Van't Hoff plot of K_{eq} versus 1/T significantly deviates from a linear line, the $R_{PPII/\beta}$ value should be readjusted and the above calculations are repeated until (1) the isodichroic-point-criterion is satisfied and (2) the linearity of the Van't Hoff plot is observed. This provides us the entropy and enthalpy differences between the PPII and β-strand conformers. Since we have estimated P_{PPII} and P_{β}, Eq. (1) with experimentally measured *J*-coupling constants allows one to determine the two reference coupling constants, J_{PPII} and J_{β} (see Table I). Then, the T-dependent functions in Eq. (8), which were obtained by iterating the SC-SVD calculations, finally correspond to the T-dependent populations (mole fractions) of the two conformers. In Figure 6, the corresponding eigenspectra of the PPII and β-strand conformers of the Ser(P) and the Thr(P) at pH = 3.0 and 8.0 are shown. The PPII eigenspectra show a strong negative peak at 195 nm and a weak positive peak at around 220 nm in the cases of the Ser(P) in pH = 3.0 and 8.0 solutions and the Thr(P) solution at pH = 3.0. In contrast, the β-strand eigenspectra have a broad negative peak in the wavelength range from 200 to 230 nm. These are characteristic features of the PPII and β-strand eigenspectra and they are fully consistent with the corresponding eigenspectra obtained from the T-dependent NMR and CD studies of trialanine in aqueous solutions. Here, it should be noted that the CD intensity of the β-strand is large at the wavelength of 222 nm. Thus, the notion that the CD intensity at 222 nm should originate from α-helical polypeptides is not always valid in the cases of the short peptides with significant β-strand population.

Temperature- and pH-dependent population changes

Carrying out the SC-SVD analyses of the T-dependent CD and NMR results of the phosphorylated and unphos-

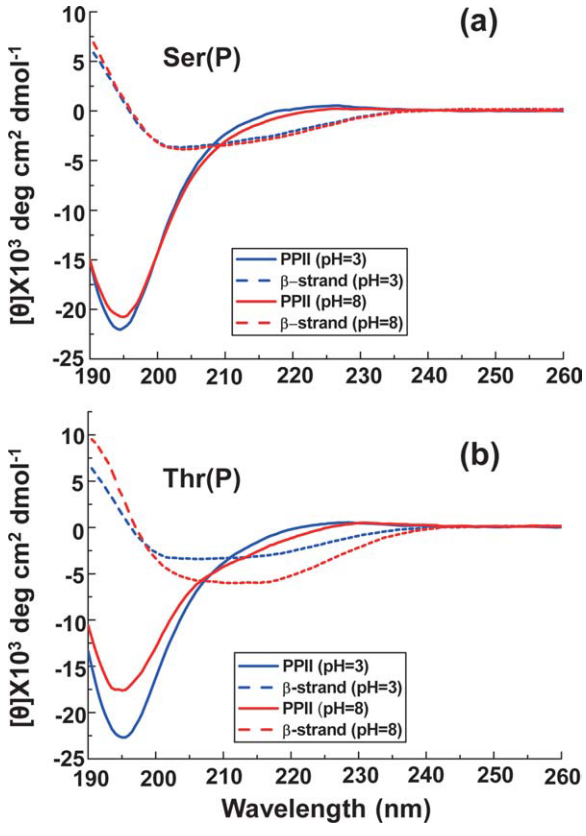

Figure 6
CD eigenspectra of PPII and β-strand conformers of the Ser(P) and the Thr(P) at pH = 3.0 and 8.0.

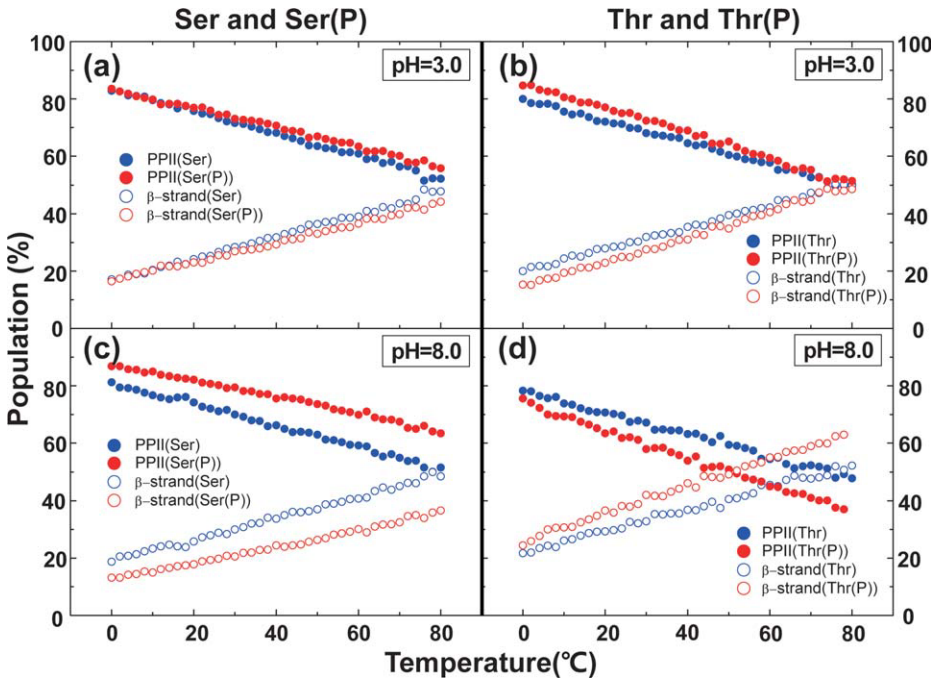

Figure 7

The percent populations of PPII and β-strand conformers of Ser, Ser(P), Thr, and Thr(P) at pH = 3.0 and 8.0.

phorylated serine and threonine peptides, we obtained the relative PPII populations. At a lower pH (= 3.0), the PPII population of the Ser(P) is almost the same with that of the unphosphorylated one [see Fig. 7(a)]. As temperature is increased, the PPII population decreases, indicating that the PPII (β-strand) conformation is enthalpically (entropically) favored over the β-strand (PPII) conformation.[11,17,20,21] Such T-dependence of the PPII population is also found in all the other cases studied here.

An important observation to be noted is that the PPII population of the Ser(P) at a higher pH (= 8.0) is significantly larger than that of its unphosphorylated one [Fig. 7(c)]. From the NMR studies, we already showed that the dianionic phosphate exclusively interacts with the N—H_{Ac} group. Such intramolecular H-bonding interaction enhances the PPII conformational propensity of the serine peptide. However, the phosphorylation of the threonine peptide makes the PPII population smaller than the unphosphorylated one at pH = 8.0 [Fig. 7(d)]. This suggests that, due to the additional intramolecular H-bonding interaction between the dianionic phosphate and the N—H_{Am} hydrogen atom of the Thr(P), the PPII propensity of the threonine residue is reduced upon its phosphorylation at pH > pK_{a2}.

An immediate question raised is why the phophate in Ser(P) forms an H-bond only with the acetyl N—H, whereas that in Thr(P) can make H-bonds with both the acetyl and amide N—H groups. To address this issue, it should be noted that the phosphate group in Ser(P) and Thr(P) even at a high pH (=8.0) can exist two distinctively different H-bond forms. As mentioned throughout this article, it can make a direct intramolecular H-bonding interaction with a backbone N—H group. However, the phosphate can also be fully solvated by surrounding water molecules. In the latter case, the phosphate does not form any intramolecular H-bonding interaction. Therefore, even for the dianionic phosphate at pH > pK_{a2}, there are these two phosphate species that are in dynamic equilibrium state. In another words, these two mechanisms, that is, intramolecular H-bond with neighboring N—H groups versus intermolecular H-bond with solvent water, compete with other. Now, let us re-examine the NMR chemical shift changes of the N—H_{Ac} and N—H_{Am} protons in Ser(P) and Thr(P) (see Figure S3 in Supporting Information) with respect to pH. The chemical shift of the N—H_{Ac} proton in Ser(P) changes by about 0.4 ppm upon the second ionization of the phosphate, whereas that in Thr(P) does by about 0.6 ppm. This suggests that the phosphate in Thr(P) can form quite strong intramolecular H-bonding interaction with the N—H_{Ac} group in comparison to that in Ser(P). That is to say, the phosphate group in Ser(P) is sufficiently flexible enough to make strong H-bonding interactions with surrounding water molecules, that is, increasing the population of fully solvated phosphate group. Due to the same reason, the phosphate in Ser(P) is not available for the intramolecular H-bond with the N—H_{Am}, which is the weakest H-bond among them. In stark contrast, the additional methyl group at the C_β-position of Thr(P) reduces

Table II
Enthalpy and Entropy Differences (in kcal/mol) Associated with the Conformational Transition from PPII to β-Strand Conformations

	ΔH *(kcal/mol)*	TΔS *(kcal/mol) at 300 K*
Ser(P) (pH = 3.0)	3.18	2.54
Ser(P) (pH = 8.0)	3.15	2.33
Thr(P) (pH = 3.0)	4.07	3.45
Thr(P) (pH = 8.0)	3.80	3.54
Ser (pH = 3.0)	3.56	2.97
Ser (pH = 8.0)	3.13	2.78
Thr (pH = 3.0)	3.31	2.82
Thr (pH = 8.0)	3.22	2.77

These values were obtained from the self-consistent singular value decomposition analyses of the T-dependent NMR and CD experimental results.

the side-chain flexibility so that the preference of the phosphate group to form intramolecular H-bonds with the N—H groups is increased with decreasing the relative population of phosphate fully solvated by water. At the moment, we, however, cannot determine the relative populations of the fully solvated phosphate, the phosphate H-bonded with N—H_{Ac}, and that with N—H_{Am} in Ser(P) and Thr(P). Nonetheless, it is believed that the detailed population distribution of these three forms of phosphate is affected by the presence of the C_β-methyl group. To examine the validity of this conjecture, we are currently performing extensive quantum chemistry calculations with explicit or implicit solvent model.

In Table II, the estimated enthalpy and entropy differences, that is, $\Delta H = H_\beta - H_{PPII}$ and $\Delta S = S_\beta - S_{PPII}$, are summarized. The PPII conformation is enthalpically more stable by about 3 to 4 kcal/mol than the β-strand conformation. However, the β-strand conformation is entropically more stable by about the same amount of energy (at 300 K) than the PPII conformation. In Figure 8, the thermodynamic properties associated with the PPII → β-strand conformational transitions are summarized. Here, it is interesting to notice that the enthalpic and entropic energy differences are not small in comparison to the thermal energy *RT* at 300 K as well as to a typical H-bond energy. However, due to the cancellation between the enthalpic and entropic contributions, the Gibbs free energy difference ΔG becomes comparable to the thermal energy. Consequently, both conformers appear to have quantitatively similar populations at physiological condition. Such an enthalpy-entropy compensation effect has been found in a number of chemical reactions and physical processes including protein folding-unfolding.[13,22–29]

ϕ angles of PPII and β-strand conformations

The byproducts of the above SC-SVD analyses of the T-dependent NMR and CD experimental results are the two reference coupling constants J_{PPII} and J_β given

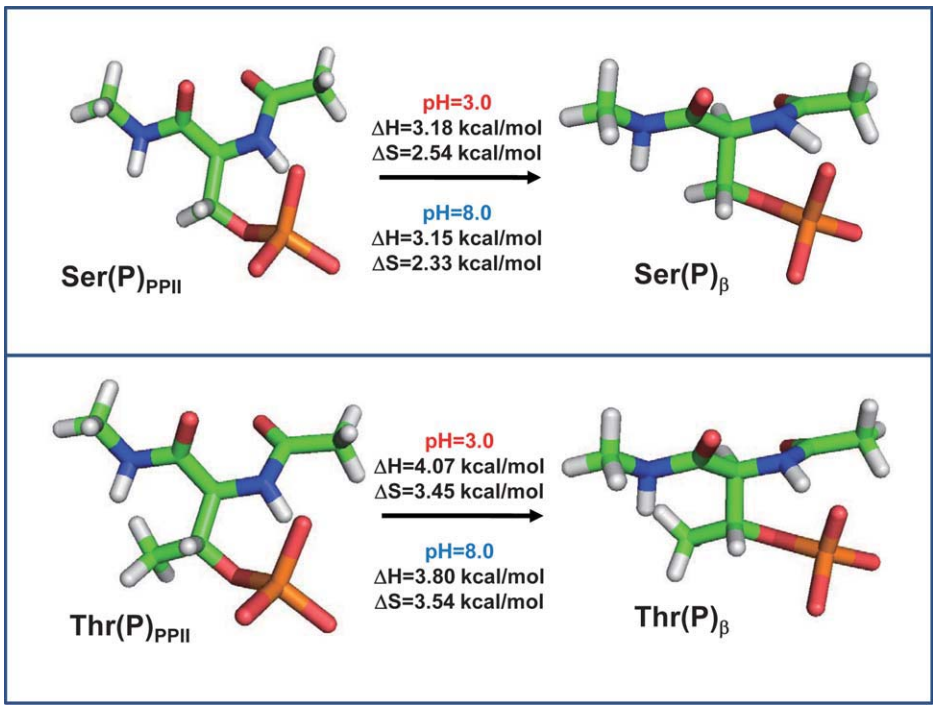

Figure 8
Difference enthalpy and entropy associated with the conformational transition from PPII to β-strand conformers of Ser(P) and Thr(P) at pH = 3.0 and 8.0.

in Table I, which could not be independently measured experimentally. From these values, one can directly estimate the dihedral ϕ angles of the ensemble average PPII and β-strand conformations, using the corresponding Karplus equation, $J(\phi) = A\cos^2(\phi - 60) + B\cos(\phi - 60) + C$.[30,31] Here, the A, B, and C parameters reported in Ref. [31] are used. Thus determined ϕ angles of the PPII and β-strand conformers of the serine and threonine peptides in solutions at pH 3.0 and 8.0 can also be found in Table I. Interestingly, the ϕ angles of the PPII and β-strand conformers vary depending on the residue and pH. For instance, the ϕ angles of the PPII conformers are in the range from -62° to -86°, and those of the β-strand conformers are from -148° to -162°. This suggests that the PPII and β-strand structures, more specifically their average ϕ angles, of a given peptide are not necessarily identical to those of the other peptides consisting of different amino acids. That is to say, the ϕ and ψ angles of a given secondary structure, for example, PPII and β-strand, have heterogeneous distribution depending on the constituent amino acid residues. Such residue-dependencies of J_{PPII} and J_β were investigated by Kallenbach and coworkers before.[32] They considered a series of host-guest pentapeptides, GGXGG, where X is one of the 18 amino-acids (Gly and Pro excluded). From the coil library of Avbelj and Baldwin, they were able to determine the ϕ angles of PPII and β-strand.[33] Then, using the Karplus equation with parameters reported by Vuister and Bax,[34] the reference coupling constants were calculated. They showed that the J_{PPII} value varies from 4.81(Ala) to 6.63(Asn) Hz and that the J_β value from 8.97(Leu) to 9.86 (Phe and Trp) Hz. Our estimated J_{PPII} and J_β values of the unphosphorylated and phosphorylated serine and threonine peptides are quantitatively similar to these values.

SUMMARY

The phosphorylation effects on the peptide backbone conformations of serine and threonine peptides have been studied by analyzing the temperature-dependent NMR $^3J(H_N,H_\alpha)$ coupling constants and CD spectra of the phosphorylated serine and threonine peptides. The populations of the PPII and β-strand conformers were quantitatively determined by using the self-consistent singular value decomposition method. In addition, we were able to determine the reference NMR coupling constants and the eigenspectra of the two conformers. As temperature is increased, the PPII (β-strand) populations of both the phosphorylated and unphosphorylated serine and threonine peptides decrease (increase), because they are entropically (enthalpically) disfavored. We also found that the PPII propensity of the serine peptide increases upon phosphorylation of its hydroxyl group. However, the effect on the threonine peptide is different from that of the serine peptide due to the methyl group on the C_β atom of the threonine side-chain, which seems to steer the intramolecular H-bonding propensity of the side-chain phosphoryl group by controlling the side-chain flexibility and the relative population of fully solvated phosphate group. We anticipate that such subtle differences in the phosphorylation effects on the peptide backbone conformations of serine and threonine peptides are important in understanding the phosphorylation-induced conformational control of peptides and proteins such as phosphorylation-specific prolyl isomerization in signal transduction.

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