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Effect of Phosphorylation on Peptidyl-Prolyl Imide Bond *cis/trans* Isomerization of Peptides With Xaa-Pro Motif *

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Abstract The peptidyl-prolyl imide bond *cis/trans* isomerization of Xaa-Pro motif in the peptide and protein plays an important role to influence their conformation and function. Here, a series of model peptides including phosphorylated and its unphosphorylated counterparts were designed and synthesized. Preliminary ^{1}H NMR experiments and molecular dynamics (MD) simulation were used to analyze the peptidyl-prolyl *cis/trans* imide bond isomerization. The data indicated that the side-chain O-phosphorylation of the Xaa residues preceding proline affected evidently the isomerization and thereby regulated the peptides conformations. The charges of the phosphate moiety as well as their steric effects might be the driving force for the conformational changes of these phosphopeptides. Moreover, the obtained most stable multiple configurations and their statistic *cis/trans* concentration distribution in MD simulation were basically consistent with the NMR experiments, which demonstrated that phosphorylation increased the *cis* conformation of the peptide and the maximum *cis* ratio is given while the phosphate group has no negative charge.

Key words peptidyl-prolyl imide bond, isomerization, phosphorylation, *cis/trans* ratio, molecular dynamics

Proline residue plays a unique role in peptide and protein structure through the conformational restriction introduced by its cyclic side chain. The small difference in free energy between the *cis* and *trans* conformations (Figure 1) of the peptidyl-prolyl bond allows these two conformations to be significantly populated [1]. This kind of *cis/trans* conformational isomerization puts kink into a polypeptide chain because of its rotationally hindered peptidyl-prolyl bond, just like a molecular switch, which has been widely accepted to be the reason for slow steps in protein folding reactions. In addition, this isomerization is believed to change the conformation of proteins and then to change their functions, including the denature and renature of proteins[2~8].

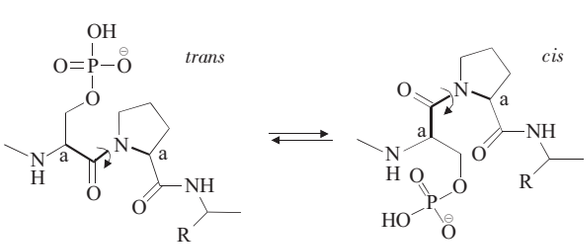

Fig. 1 Peptidyl-proyl conformation of *cis/trans* isomers

Recently, study on the factors affecting the *cis/trans* isomerization of peptidyl-prolyl imide bond has received both experimental and theoretical attention. It was found that the *cis/trans* ratio of peptidyl-prolyl bonds can be affected by many factors. The structural property of the amino acid residues (Xaa) preceding proline in polypeptide, which is usually defined as the local side-chain effects, is proposed to be the leading one[9]. O-phosphorylation of the side-chain on Xaa (Xaa= Ser/Thr/Tyr) may be one of the most important factors; and the study in this field has already shed light on the mechanism of some

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conformational dependent illness [10]. The reversible phosphorylation of protein on amino acid residues preceding proline (Ser/Thr/Tyr-Pro), the well-known posttranslational modification way, plays a key role in almost all biochemical processes, from control of catabolism/metabolism through signal transduction to growth and gene expression [11]. Although phosphorylation has been proposed to regulate the function of a protein by inducing conformational changes, it is still far from a whole picture about what phosphate additions actually do and how the functions of phosphoproteins are coordinated. The majority of the mechanisms described so far, however, show phosphorylation works through changing protein conformation in a manner that requires the introduction of the phosphoryl group into an existing network of interactions within the context of a globular protein with well defined tertiary structure[12~14]. It was also suggested that the presence of a phosphate group might exert direct effects as well as indirect effects on protein conformation, for example, changing the preferred dihedral of the peptide or other adjacent bonds.

To look for such direct effects of phosphoryl groups in the absence of tertiary interactions, several models have been proposed to explain how phosphorylation of Ser/Thr side chains is able to regulate the function of a protein[15]. However, direct identification of proline switches is challenging, as the isomerization itself is invisible in most biochemical methodologies. As we know, nuclear magnetic resonance (NMR) spectroscopy has become one of the best available methods to directly detect the *cis*/*trans* peptidyl-prolyl isomerization in a folded protein. Although detecting and characterizing the minor *cis* forms is proven to be less straightforward because of the sensitivity of the instrument, the assignment of short model peptides can be easily carried out now according to the sequential assignment procedure based on NMR spectra[16].

In this paper, based on the Fmoc-strategy, Ac-Ala-Xaa-Pro-Lys-NH-Np model compounds were designed (typical chemical structure was shown in Figure 2) and a series of phosphorylated/ unphosphorylated tetrapeptides were subsequently synthesized with the solid-phase method. Due to the advantage of NMR in detecting the prolyl isomerization in short peptides in solution, we found that the phosphoryl group covalently attached to Ser/Thr/Tyr-Pro motif exerts a direct effect on the preferred backbone conformation and causes the *cis*/*trans* population diversion. Meanwhile, based on the efforts of our laboratory in the theoretically computational field[17~19], we selected Ac-Ala-Xaa-Pro-Lys-NH-Me model to perform the molecular dynamics simulation in an attempt to establish the side-chain O-phosphorylation effect in regulating the conformations of peptides. On the whole, the simulation results matched the NMR experimental data well and further proved the effect of phosphorylation on the peptidyl-prolyl imide bond *cis*/*trans* isomerization that acts as a molecular switch.

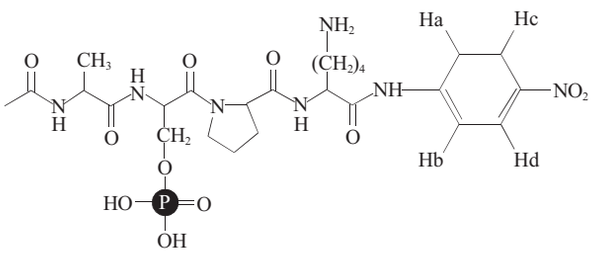


Fig. 2 Protons on benzyl ring tested

1 Experimental section

1.1 Peptide synthesis and purification

The peptides were synthesized with global phosphorylation method using solid-phase Fmoc-strategy. Rink Imide-Am resin with loading capacities of 0.52 mmol/g and a 4 times excess of each Fmoc-protected amino acid were used for the synthesis. The Fmoc group was removed before each coupling step by reaction with 20% piperidine in DMF for 5 min and 20 min. Entering amino acids were preactivated with HOBt (1-hydroxybenzotriazole, 1.5 mmol), HBTU (O- benzotriazole- N, N- N′-N′-tetramethyluronium hexafluorophosphate, 1.5 mmol), and DIEA (N,N'-diisopropyl ethylamine, 3.0 mmol) in DMF (4.0 ml) for 5 min, and couplings were run for 1.5～2 h. The whole peptide was conjugated with the phosphorylation reagents followed by oxidation to obtain the phosphopeptides. Peptide chains were cleaved from the resin and amino acid side-chain protecting groups were removed by reacting with cleavage reagent (95% TFA) for 2 h. Put the filtrate into the dry ether and the deposit of crude products were obtained and characterized with ESI-MS.

Crude peptides were purified by semi-preparative high-performance liquid chromatography (HPLC) on a reverse-phase 9.4 ～250 mm SB-C18 column

(ZORBAX 30 nm pore size) using a Waters (Milford, MA, USA) 600 HPLC system equipped with a Waters 2487 dual wavelength UV absorbance detector. The purity, as indicated by HPLC and ^{1}H NMR, was higher than 98% for all the peptides listed in Table 1. The purified peptides were lyophilized and stored at −7℃ before used.

Table 1 Chemical shift of aromatic protons of NH-Np group of the peptide Ac-Ala-Xaa-Pro-Lys-NH-Np

Entity	Xaa	δ (peaks in downfield)				δ (peaks in upfield)			
		trans		*cis*		*trans*		*cis*	
1	L-Ser	8.182	8.162	8.081	8.063	7.626	7.612	7.578	7.562
2	L-Thr	8.160	8.152	8.145	8.128	7.612	7.597	7.592	7.577
3	L-Tyr	7.921	7.906	7.804	7.794	7.398	7.384	7.302	7.288
4	L-Ser(PO(OH)$_2$)	8.179	8.165	8.089	8.077	7.646	7.631	7.608	7.593
5	L-Ser(PO(OH)(OMe))	8.197	8.182	8.088	8.072	7.668	7.652	7.634	7.618
6	L-Ser(PO(OMe)$_2$)	8.124	8.109	8.018	8.003	7.580	7.564	7.537	7.522
7	L-Thr(PO(OH)$_2$)	8.205	8.190	8.180	8.165	7.661	7.646	7.622	7.048
8	L-Thr(PO(OH)(OMe))	8.163	8.149	8.119	8.110	7.640	7.626	7.590	7.574
9	L-Thr(PO(OMe)$_2$)	8.395	8.380	8.254	8.242	7.899	7.885	7.850	7.835
10	L-Tyr(PO(OH)$_2$)	8.147	8.131	8.058	7.946	7.598	7.582	7.565	7.549
11	L-Tyr(PO(OH)(OMe))	8.109	8.095	7.938	7.926	7.598	7.584	7.534	7.519
12	L-Tyr(PO(OMe)$_2$)	8.143	8.126	8.043	8.029	7.624	7.609	7.556	7.540

1.2 Nuclear magnetic resonance (NMR)

Samples for ^{1}H NMR experiments were prepared by dissolving the peptide in 90% H_2O/10% D_2O at 4～6 mmol/L concentrations. The pH of the sample was adjusted by adding KOH or HCl (0.5 and 0.05 mol·L^{-1}) to 6.5. ^{1}H NMR spectra were recorded on a Varian INOVA-600 spectrometer with a proton resonance frequency at 599.8 MHz. The probe temperature was 298K. Spectra were referenced to the methyl resonance of TMS at 0.000.

1.3 Molecular dynamics (MD) simulations

Conformational searches and molecular dynamics simulations were performed on each of the peptides with Insight Ⅱ version 7.0 on SGI O2 R10000 workstation. The Insight Ⅱ implementation of AMBER (version 4.0) all-atom force filed was used [20,21]. For solution-phase calculations, the explicit model for water was used. The aqueous solution for a peptide was built by immersing it into an equilibrium water box (initial dimensions approximately 3.0 nm×2.5 nm×2.5 nm with around 1 200 water molecules) and deleting overlapping water molecules that were within 0.25 nm of the peptide. The TIP3P water model was used to represent water molecules. For simulations in explicit water, a cutoff of 0.8 nm was used to evaluate non-bonded interactions. In our case, no counterions were added because adding ions to balance the charge in this system might lead to very high effective salt concentration in our case, which could affect the stability of the system[22]. Imide bonds were required to be *trans* due to the improbable occurrence of *cis* imide in low energy structures except in the case of proline whose imide bond was purposefully sampled and accepted with either *cis* or *trans* geometry in the conformational searches.

Conformational searches were performed using the MD/SD (Molecular Dynamics / Stochastic Dynamics) conformational search method. For each search, a time step of 1.0 fs was used for the MD part of the algorithm. The total simulation time was 1 000 ps and samples were taken at 10 ps intervals, then 100 conformations were generated and minimized using the truncated Newton-Raphson method implemented in Insight Ⅱ until the gradient was less than 0.05 (kJ/mol)/Å^{-1}.

2 Model design

Neighboring amino acid side chains around the Xaa-Pro motif need to be overly bulky to prevent steric clashes with the phosphoryl group. To study the influence of charge, the amino and carboxyl terminal need to be blocked through acetylation and amidation, respectively. In this article, the model tetrapeptides Ac-Ala-Xaa-Pro-Lys-NH-Np were chosen to undergo the following study. Xaa-Pro motif (Xaa =

Ser/Thr/Tyr) was extended by amino acid residue in each terminal. Despite disobeying to the above-mentioned law, the downstream residue lysine from the Xaa-Pro motif was put into this sequence in consideration of the further dynamic experiments, for the imido peptide bond formed between it and the 4-nitroanilide group was liable to be specifically hydrolyzed in chymotrypsin solution. The O-phosphorylation was occurred on the Xaa side chain in three forms: Xaa-P($O(OH)_2$), Xaa-P($O(OH)(OCH_3)$), and Xaa-P ($O(OCH_3)_2$), which correspond to three kinds of charge states at phosphate group in solution with a certain pH value (4.7 in our case): more than one negative charge (between 1 and 1.5) averagely, one negative charge, electroneutral, respectively. Thus, the influence of different charges on the peptides conformation could be estimated. Besides, by increasing the pH value to about 6.5, Xaa-P($O(OH)_2$) nearly carries two negative charges.

One of intentions of the introduction of 4-nitroanilide group, just like the acetyl group, was to eliminate the terminal activity of peptides to rule out the unnecessary electrostatic interaction. Another purpose was that the 4-nitroailide facilitates the measurement of the *cis*/*trans* population in those peptides.

The four aromatic protons on benzyl ring, whose symmetrical properties make a characteristic peak in NMR spectrometry that can be distinguished easily, were used for analysis. The protons labeled *a* and *b*, have the same chemical shift, nearly from 7.5 to 8.3. Similarly, *c* and *d* are also equivalent protons and have the same value in this shift range. Although the peaks of NH protons appeared simultaneously in this area, the doublet feature makes them easy to be assigned. The determination of the conformer ratio was based on the integrated peak intensities of these aromatic proton signals.

3 Results and discussion

3.1 Chemical shift assignments of the NMR spectra

Twelve peptides were studied with ^{1}H-NMR and a complete assignment of the signals for the *cis* isomer was restricted by low *cis* contents in combination with considerable overlapping of *cis* and *trans* signals. Because the rate of *cis* to *trans* isomerization is slow (typically <0.004 s^{-1})[23], two sets of resonances were seen for many of the residues. Best signal dispersion for *trans* and *cis* isomers is usually obtained in the NH regions of the spectra, because no spectral overlap of imide protons is observed for either the unphosphorylated or the phosphorylated peptides in this area. The NH part of the spectrum was shown in Figure 3. The total spectrum of the peptide displayed that the peaks in aliphatic region with a shift range between 0.8 and 4.2 were heavily crowed.

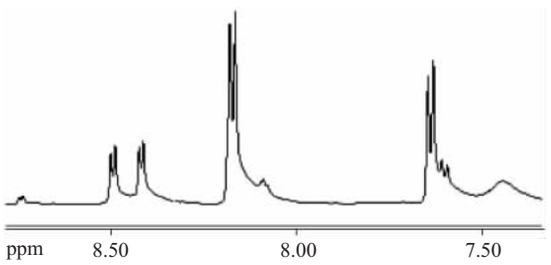


Fig. 3 NH part of the 1D-NMR spectrum of the peptide Ac-Ala-Ser-P (O (OH)$_2$) -Pro-Lys-NH-Np

The chemical shift values were so similar that the *cis* isomer-derived signals suffered from signal overlap, precluding definite signal quantification. In the range between 6.5 and 8.4, the peaks can be separated clearly and display a strong isomer-related signal splitting. Because of the existence of nitro group, the peaks of aromatic protons on NH-Np has a slight downfield shift by 0.2, while the peaks of protons on the benzyl ring of tyrosine are in the upfield. So the peaks at 6.48, 6.49, 6.81, 6.82 correspond to the benzene protons of tyrosine, the peaks in 7.38, 7.40, 7.91, 7.92 correspond to the benzene protons of NH-Np group. According to the literature[12], the peaks at 7.72, 7.86, 7.89, 8.19 are assigned as NH proton. Because the solvent used in the experiment was 90% H_2O in D_2O, the active hydrogen atoms did not exchange with deuterium, the peaks at 6.53, 6.85 are assigned as OH and NH_2 protons. The aromatic proton in the *cis* isomer have a larger upfield shift relative to the *trans* isomer, thus the peaks at 7.29, 7.30, 7.79 and 7.80 should correspond to benzene protons on NH-Np group in *cis* form, while the peaks in 7.38, 7.40, 7.91, 7.92 are in *trans* form. In comparison with its unphosphorylated analogues, the chemical shift of all the phosphorylated peptides varied differently to some extent, and the chemical shift changes observed for many residues resonances upon conversion from the *cis* to *trans* isomer in these twelve peptides are consistent with those trends predicted for random coil resonances[1,24]. Complete

resonance assignments are made for the protons on benzyl ring of NH-Np group of the twelve peptides, as shown in Table 1.

3.2 Effect of Phosphorylation on *cis/trans* isomerization

Prolyl *cis*/*trans* isomerization is a slow process with relaxation times of the order of ten seconds in unstructured peptides. In reported unstructured peptides [9] with the sequence Ac-Ala-Xaa-Pro-Ala-Lys-NH_2 (where Xaa is any amino acid), the residue before proline was the primary determinant of the *cis*/*trans* ratio. However, the variation in the ratio over different peptides was very small, except for peptides in which Xaa was an aromatic residue, which has higher percentages of *cis* isomers. In other studies of short Ser/Thr-Pro-containing peptides, 5%～20% *cis* isomer was detected both before and after phosphorylation. In this work, based on the sequence Ac-Ala-Xaa-Pro-Lys-NH-Np, the effect of phosphorylation on peptidyl-prolyl *cis*/*trans* isomerization was explored. The *cis*/*trans* peaks integral of the benzene protons of NH-Np group were listed in Table 2 and a typical diagram was given as Figure 4.

Table 2 Peak integral of aromatic protons of NH-Np group in the peptide Ac-Ala-Xaa-Pro-Lys-NH-Np

Entity	Xaa	The peak intensities of *cis*/*trans* isomers in downfield			The peak intensities of *cis*/*trans* isomers in upfield			Average *cis* content /%
		trans	*cis*	*cis* %	*trans*	*cis*	*cis* %	
1	L-Ser	4.23	0.28	6.20	3.17	0.27	7.80	7.00
2	L-Thr	36.04	5.80	13.90	32.78	4.05	10.90	12.40
3	L-Tyr	15.84	3.29	17.20	14.24	2.79	16.40	16.70
4	L-Ser($PO(OH)_2$)	35.88	9.01	20.10	18.38	4.10	18.20	19.15
5	L-Ser(PO(OH)(OMe))	20.34	5.38	20.90	12.17	3.65	23.10	22.00
6	L-Ser($PO(OMe)_2$)	26.01	8.97	25.60	24.70	12.00	32.70	29.15
7	L-Thr($PO(OH)_2$)	0.90	0.13	12.60	1.09	0.20	15.50	14.05
8	L-Thr(PO(OH)(OMe))	3.42	0.56	14.10	2.22	0.32	12.60	13.35
9	L-Thr($PO(OMe)_2$)	23.46	3.51	13.00	20.22	3.47	14.60	13.80
10	L-Tyr($PO(OH)_2$)	21.26	3.62	14.50	26.88	6.64	19.80	17.15
11	L-Tyr(PO(OH)(OMe))	12.23	3.17	20.60	11.88	3.86	24.50	22.55
12	L-Tyr($PO(OMe)_2$)	17.38	4.78	21.60	19.23	6.93	26.50	24.05

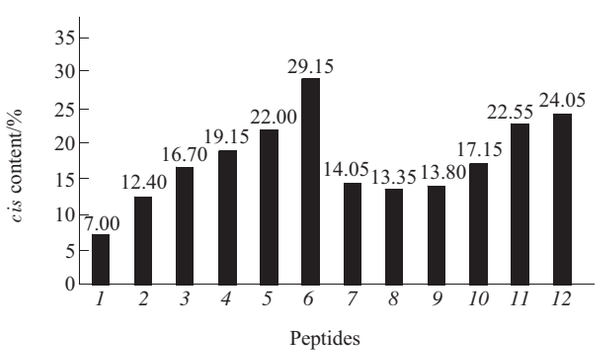


Fig. 4 The *cis* content of different peptides

It was generally found that the larger the side chain group was, the higher the *cis* content of the peptides was. Comparing the three unphosphorylated peptides 1, 2, 3, with the value of 7.0%, 12.4% and 16.7% respectively, the distribution for *cis* content was in the following sequence: L-Ser < L-Thr < L-Tyr. For the sequence with Ser/Tyr-Pro motif, the *cis* contents also grew up with the augment of the phosphate moiety. The observation was essentially consistent with the steric hindrance effect published in the earlier research[9]. Studies of small linear peptides[9,19] showed that the aromatic residues, Tyr, Phe, His, and Trp, in the position preceding proline, are more efficient than other natural occurring amino acids in stabilizing the *cis*-imide bond conformation, a property proposed to be due to the steric bulk of their side chains. In our work the aromatic residue tyrosine gives the higher fraction of *cis* isomer among these peptides. Comparing the phosphorylated peptide with its unphosphorylated one, apparently the former got more *cis* content. The existence of phosphoryl group could obviously increase the fraction of *cis* isomer of the peptide. However, the peptides series containing the Thr-Pro motif seems not to be significantly affected by

the phosphorylation and changes a little in *cis* population. Schutkowski and co-workers[25] reported that the prolyl isomerization of the phosphopeptides was slower compared to the corresponding unphosphorylated compounds. This effect was more pronounced for peptides containing phosphothreonine residue preceding praline than others. The dynamic stability might be responsible for the result.

Another interesting observation was that phosphorylation influences the *cis* population to different extent of the peptides containing different Xaa-Pro motif. Peptide series with Ser-Pro motif have the largest variation amplitude, as shown in Figure 5. The *cis* content of the unphosphorylated form containing Ser-Pro motif, was only 7.0%, which was the least one among all these twelve peptides. Whereas, the *cis* content went up to 19.15% when the serine was phosphorylated, and it could reach the top value by 29.15% in the other phosphorylated forms. The peptides series with Tyr-Pro motif also obtained a visible increase of their *cis* population after phosphorylation. For peptides series with Thr-Pro motif, difference in the *cis* content between the phosphorylated and unphosphorylated was very small, which indicated that phosphorylation of the Thr-Pro motif was fairly insensitive to the *cis*/*trans* isomerization of the corresponding prolyl bond. What the almost unchanged fraction of its *cis* isomer indicated remained to be ambiguous. Mayr's group[26] pointed out that the *cis*/*trans* isomerization of the Tyr-Pro bond was two times faster in the tetrapeptide and the rate constant for the Ser-Pro bond isomerization was quite similar, Schutkowski[25] also proposed that peptides containing phosphothreonine preceding proline had the slower prolyl isomerization rate. Such a situation was possibly ascribed to this distinction between their isomerization kinetics. In addition, the increase of the *cis* isomer after phosphorylation might partially due to the destabilization of the structure in the *trans* isomer by the increased distance and the subsequent disappearance of reciprocal hydrogen bonds. Therefore, a molecular dynamic simulation method might be more helpful to deeply probe the details. Further investigation about this problem is under way now in our laboratory.

As depicted in Figure 5, it is concluded that the charge state of the phosphate moiety affected the *cis*/*trans* isomerization evidently. According to the reported pKa_1 (~0.9) and pKa_2 (~6.2) values for the R-(PO(OH)$_2$), under such conditions the net negative charges at the phosphate group would be in the following sequence: R-(PO(OH)$_2$) > R-(PO(OH)(OMe))> R-(PO(OMe)$_2$). The effect of the different phosphoryl charge state on the *cis* content of the peptide was compared in the following order: R-(PO(OH)$_2$) < R-(PO(OH)(OMe)) < R-(PO(OMe)$_2$). Especially in the peptide in which Xaa=L-Ser and L-Tyr, the effect of the phosphoryl group with no negative charges was higher than those with negative charges. This may indicate that phosphorylation results in a specific increase or decrease in the *cis*/*trans* ratio due to highly localized changes in the pThr/pSer/pTyr prolyl bond caused by steric or electrostatic effects of the phosphate group, for example, the hydrogen bonds. Hydrogen bonds between nearby imide protons and the phosphate group appear to be the driving force for these structural changes. Such alterations also be related with changes in enthalpy associated with addition of the negative charge. However, in view of the fact that the steric effects of phosphorylation also is in the same sequence, it is difficult to make sure the effects caused by charges. Experiments performed at pH 6.7, at which R-(PO(OH)$_2$) nearly carries two negative charges, pronounce a lower *cis* content than those at a pH value of 4.7, at which R-(PO(OH)$_2$) only carries less than 1.5 negative charges averagely. Therefore, it can be preliminarily concluded that the carried negative charges could decrease the *cis* content to some extent. The following theoretical exploration

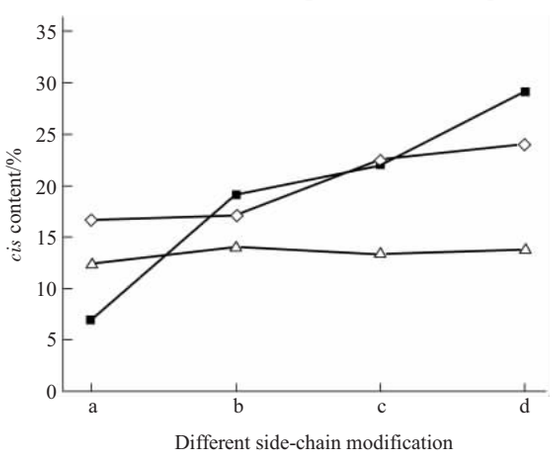


Fig. 5 Different effect of phosphorylation on the *cis* content of the peptide based on different Xaa. a, b, c, and d correspond to unphosphorylated form, PO(OH)$_2$, PO(OH)(OMe), and PO(OMe)$_2$

■—■: Ser-Pro; △—△: Thr-Pro; ◇—◇: Tyr-Pro.

should be carried out to further explain the actual mechanism for these changes.

3.3 Molecular dynamics simulation

To reduce the computational intensity, model tetrapeptide Ac-Ala-Xaa-Pro-Lys-NHMe of which we substituted smaller group —NHMe for —NHNp, was selected to undergo the following simulating investigation. Integral charge was selected which was convenient for the simulation: Ser-P(O)(O^-)$_2$, Ser-P(O)(O^-)(OCH_3) and Ser-P(O)(OCH_3)$_2$. The most stable conformations were obtained and the simulating results were summarized in Tables 3 and 4, which indicated that all four located most stable *trans* conformations of these four peptides are seemingly open structures and show a very similar spatial arrangement. However, in the four most stable *cis* conformations, based on the interproton distance between the α carbon of the *i* (Ala) and *i*+3(Lys) residue of peptide Ac-Ala-Ser (PO (OMe)$_2$)-Pro-Lys-NH-Me (*cis*-ApSPK, as shown in Figure 6) and Ac-Ala-Ser-Pro-Lys-NH-Me (*cis*-ASPK), a β Via turn structure was found according to the references[27,28]. Therefore, combining with the experimental data mentioned above, we get a conclusion that the zero anionic state facilitates the formation of a regular conformation after the phosphorylation.

Table 3 Dihedral angle and energies for each conformation of peptide Ac-Ala-Xaa-Pro-Lys-NH-Me

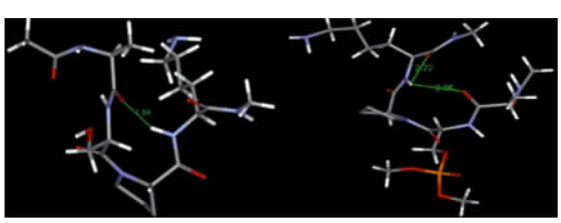
Entity	Xaa	Dihedral angle ω /(deg.)			The lowest energy /(kJ·mol·L^{-1})		
		cis	*trans*[1)]	*trans*[2)]	*cis*	*trans*[1)]	*trans*[2)]
1	Ser	6.08	164.74	−170.06	−1064.80	−1059.09	−1084.49
2	Ser-PO(O^-)(OMe)	−34.57	177.34	−157.91	−1316.44	−1320.97	−1312.54
3	Ser-PO(O^-)$_2$	−13.69	172.22	−175.52	−1322.42	−1313.59	−1330.92
4	Ser-PO(OMe)$_2$	19.85	176.39	−172.47	−1290.33	−1300.61	−1295.30

[1)] Dihedral angle near the -180° . [2)] Dihedral angle near the +180°.

Table 4 Interatomic distances (Å) between *i* and *i*+3 residue of the model peptide Ac-Ala-Xaa-Pro-Lys-NH-Me

Entity	Xaa	Distance between Ala-CαH and Lys- CαH			Distance between Ala-(CO)and Lys -(NH)		
		cis	*trans*[1)]	*trans*[2)]	*cis*	*trans*[1)]	*trans*[2)]
1	Ser	5.46	10.15	9.97	1.84	5.89	5.89
2	Ser-PO(O^-)(OMe)	8.47	10.01	9.73	5.78	6.56	6.08
3	Ser-PO(O^-)$_2$	6.82	6.07	10.14	5.38	6.82	6.82
4	Ser-PO(OMe)$_2$	6.39	9.92	9.50	2.82	6.39	6.68

[1)] Dihedral angle near the −180° . [2)] Dihedral angle near the +180°.



cis-ASPK *cis*-ApSPK*

Fig. 6 Structures of located most stable conformations

Hydrogen atoms except the αH on pyrrolidine ring of proline are not shown in order to retain clarity. Backbone and carbon atom are in gray color, oxygen red, phosphorus orange, hydrogen white, nitrogen blue. Hydrogen bonds are indicated by green lines. These figures were generated by use of Insight Ⅱ and processed by WebLab ViewerLite software. *Ac-Ala-Ser(PO(OMe)$_2$)-Pro-Lys-NH-Me.

By the MDSD searching process, 100 low energetic conformations for each tetrapeptide were obtained and the distribution ratio was displayed in Figure 7. The statistic results of *cis* contents for the peptides Ac-Ala-Xaa-Pro-Lys-NH-Me (Xaa = Ser, Ser-PO(O^-)(OMe), Ser-PO(O^-)$_2$, Ser-PO(OMe)$_2$) were 30% , 19% , 13% and 34% , respectively. The phosphopeptide Ac-Ala-Ser (PO (OMe)$_2$)-Pro-Lys-NH-Me with zero negative charge on its phosphate moiety got the most *cis* population among these four peptides, which was basically in good agreement with our NMR experiment of model Ac-Ala-Xaa-Pro-Lys-NH-Np (Table 5).

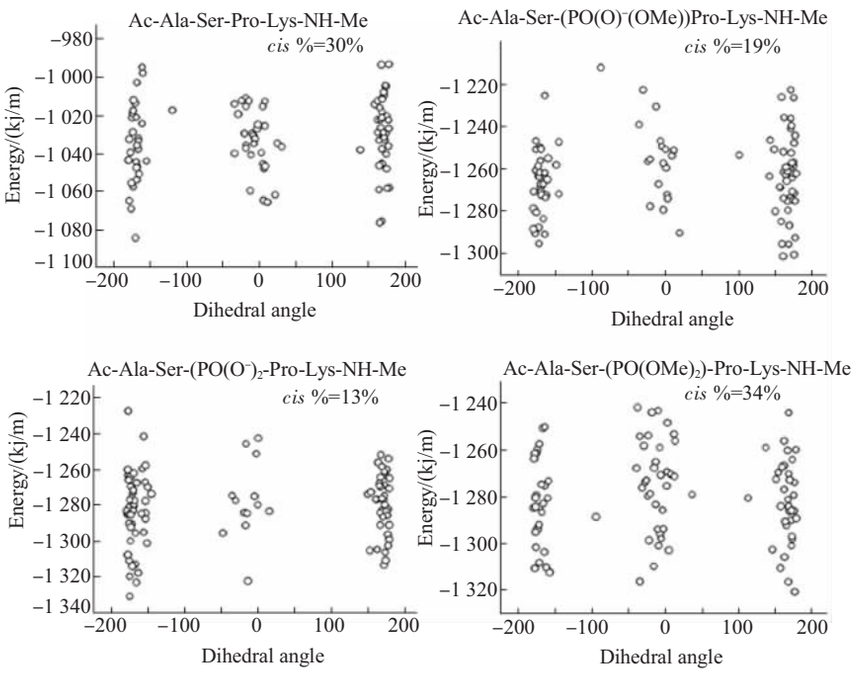


Fig. 7 Low energetic conformation distribution of four tetrapeptides

Table 5 Contrast of the *cis* content (%) between simulated data and experimental data

	The *cis* content of unphosphorylated peptide	The *cis* content of phosphorylated peptides in different charge state[3)]		
		1	2	3
Simulating data[1)]	30	34	19	13
Exp. Data[2)]	7	29.1	22.0	19.15

[1)]Simulated in the model of Ac-Xaa-Pro-Lys-NH-Me; [2)]Tested in the model of Ac-Xaa-Pro-Lys-NH-Np (Xaa=Ser). [3)]1), 2), and 3) correspond to Ser-P(O)$(OCH_3)_2$, Ser-P(O)$(O^-)(OCH_3)$ and Ser-P(O)$(O^-)_2$, respectively.

4 Conclusion

Phosphorylation on the side-chain hydroxyl group of Xaa (Xaa=Ser, Thr, Tyr) residues preceding proline in our model peptides increase the *cis* content generally. The physical changes induced by this kind of modification were obvious: steric characteristics and charges, which might alter the ability to form hydrogen bonds within the nearby space and subsequently change the *cis*/*trans* ratio equilibrium. Although the introduction of phosphate group with larger steric hindrance is likely the most decisive one among these factors mentioned above, it should be the cooperation of other factors such as charges. The simulating results also essentially supported the NMR experimental data. In summary, the studies presented here provided a structural model to study how phosphorylation could act as a molecular switch by regulating the conformation of the phosphorylated substrate. However, the exactly driving factor to crucially influence the peptides conformation remained to be further developed yet, and much more related experiments are under way in our group now.

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磷酸化对多肽中 Xaa-Pro 片段肽脯酰胺键顺反异构的影响研究 *

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摘要 多肽和蛋白质中 Xaa-Pro 片段肽脯酰胺键顺反异构对其构象与功能有重要影响. 设计合成了一系列模型多肽及其磷酸化多肽，并采用核磁共振实验和分子动力学模拟的方法，研究了所合成多肽中肽脯酰胺键的顺反异构化. 结果表明，对脯氨酸之前的 Xaa 残基进行侧链 O- 磷酸化会极大地影响该顺反异构化过程，进而调节肽链构象. 此外，磷酸化使得多肽顺式构象比例增加，且当磷酸基团不带负电荷时顺式构象所占比例最大. 同时，分子动力学模拟所得结果与核磁共振实验相一致，包括最稳定构象和顺反构象统计分布. 磷酸基团所带电荷及其空间位阻可能是影响这类磷酸化多肽构象变化的主要因素.

关键词 肽脯酰胺键，异构化，磷酸化，顺反异构比，分子动力学模拟
学科分类号 O62

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