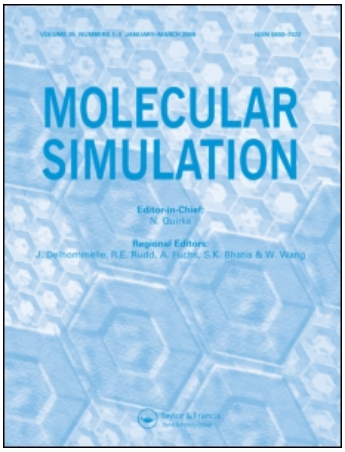

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The effect of phosphorylation on the conformation of oligo-peptides with Ser-Pro motif: a molecular dynamics simulation

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The effect of phosphorylation on the conformation of oligopeptides with Ser–Pro motif: a molecular dynamics simulation

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Model tetrapeptide system was designed to investigate the *cis*/*trans* isomerization of peptidyl-prolyl imide bond of Ser—Pro motif. To establish the side-chain *O*-phosphorylation effect in regulating the peptides conformations, molecular dynamics (MD) simulations where carried out on the designed tetrapeptides and their corresponding phosphorylated forms by MD Insight II Discovery3 approach. The most stable configurations and the statistic *cis*/*trans* concentration distribution demonstrated that the phosphorylation evidently influences the peptidyl-prolyl imide bond isomerization and works as a key effect in regulating the peptide conformations. The charge state and the site provided for the charge of the phosphate moiety might be an important key. The results also demonstrated that phosphorylation changes the *cis* conformation ratio of the peptide and the maximum *cis* value is obtained when the phosphate group has no negative charge.

Keywords: Conformational analysis; Molecular dynamics; Phosphorylation; Ser–Pro motif; *cis*/*trans* isomerization

1. Introduction

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 kinks 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].

Research on factors affecting the *cis*/*trans* isomerization of peptidyl-prolyl imide bond has become a subject of growing interest. The amino acid residues preceding proline in polypeptide are generally recognized to be the leading one, which is usually defined as the local side-chain effects [9]. Side-chain *O*-phosphorylation on Xaa is thought to be the most important factor; and the study in this field has already shed light on the mechanism of some conformational dependent illness [10]. The reversible phosphorylation of protein has come to be recognized as the prevalent mechanism by which nearly every cellular enzyme is regulated. 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 few described mechanisms showed that phosphorylation works through changing protein conformation, in a manner that requires the introduction of phosphoryl group into an existing network of interactions within the context of a globular protein with well defined tertiary structure [11–13]. It was also suggested that the 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 investigate such direct effects of phosphoryl groups in the absence of tertiary interactions, NMR experiments were carried out with short peptides containing serine, threonine, or tyrosine preceding proline in either their unphosphorylated or phosphorylated form. Although some clues have been found, the real mechanism remains

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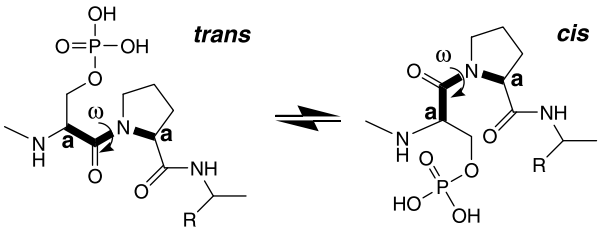

Figure 1. Peptidyl-proyl conformation of *cis*/*trans* isomers.

ambiguous yet. Therefore, a theoretical calculation study might be more helpful to deeply probe the detail.

In recent years, much interest has focused on the dynamics of isomerization of the Xaa–Pro peptide bond in various proteins. A full study of proline isomerization has been made by using empirical energy function and *ab initio* calculations [14,15]. Molecular dynamics (MD) simulation techniques have recently been demonstrated as a useful tool to study the *cis*/*trans* isomerization of proline in Staphylococcal nuclease [16–21]. In this paper, the preferred conformation and possible conformational changes induced by phosphorylation of the designed tetrapeptide and its phosphorylated analogues were investigated using molecular modeling method. The conformational analysis, including the *cis*/*trans* distribution ratio and the regulation of conformation by phosphorylation, was carried out with MD Insight II Discovery3 simulation and molecular dynamic/stochastic dynamics (MD/SD) simulation.

2. Methods

2.1 Computational technique

Conformational searches and MD 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 [22,23]. 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 $30 \times 25 \times 25$ Å with around 1200 water molecules) and deleting overlapping water molecules that were within 2.5 Å of the peptide. The TIP3P water model was used to represent water molecules. For simulations in explicit water, a cut-off of 8 Å was used to evaluate non-bonded interactions. In our cases, 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 [24]. 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 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 1000 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)/$Å^{-1}$.

2.2 NMR 2D-NOESY method

NMR sample for NOESY experiments were prepared by dissolving peptide in H_2O/D_2O 9:1 (v:v) at concentrations between 4 and 6 mM. The pH of the sample was adjusted by adding KOH or HCl (0.5 and 0.05 mol·L^{-1}) to 4.7. The data were recorded on a Varian INOVA-600 spectrometer with a proton resonance frequency at 599.8 MHz. The probe temperature was 298 K.

3. Results and discussion

3.1 Model design and the definition of the *cis*/*trans* state

The significance of the peptidyl-prolyl imide bond *cis*/*trans* isomerization has been evidenced by the discovery of highly specialized peptidyl-prolyl *cis*/*trans* isomerase (PPIase) Pin1 [25]. The crystal structure of Pin1 containing an Ala-Pro dipeptide substrate revealed a sulfate ion located 5 Å from the C_β carbon of Ala, which suggested that phosphorylated-Ser (pS) might be preferred at this site [26,27]. It was reported that the dianionic form of the Ser/Thr phosphate moiety was a good substrate specific for Pin1. Therefore, combined with our previous studies, a model tetrapeptide Ac–Ala–Ser–Pro–Lys–NHMe was selected in this article for the following study. It is well known that the charge state of the phosphate moiety might profoundly affect the peptidyl-prolyl imide bond *cis*/*trans* isomerization. Besides, as far as we know, the *O*-phosphorylation occurred on the serine side chain can cause a different charge state in solution. Therefore, in this work three phosphorylated tetrapeptides with different charges, i.e. Xaa—$P(O(OH)_2)$, Xaa—$P(O(OH)(OCH_3))$ and Xaa—$P(O(OCH_3)_2)$, were used to mimic the ionization state of the phosphate moiety in physiological conditions. At pH 4.7, the lysine side-chain should be protonated and then carries one positive charge, which is the same for all the four model peptides. Thus, we only consider the charges caused by the phosphorylation. Peptide **d** is only introduced a electroneutral phosphate group with larger steric hindrance. As for peptide **b**, just one negative charge is carried on the phosphate moiety at the studied pH. However, peptides **c** may carry more than one negative charge (between 1 and 1.5) averagely, and two sites can be provided for the negative charges, which is different from peptide **b**. Chemical structures of the designed four tetrapeptides were shown in figure 2.

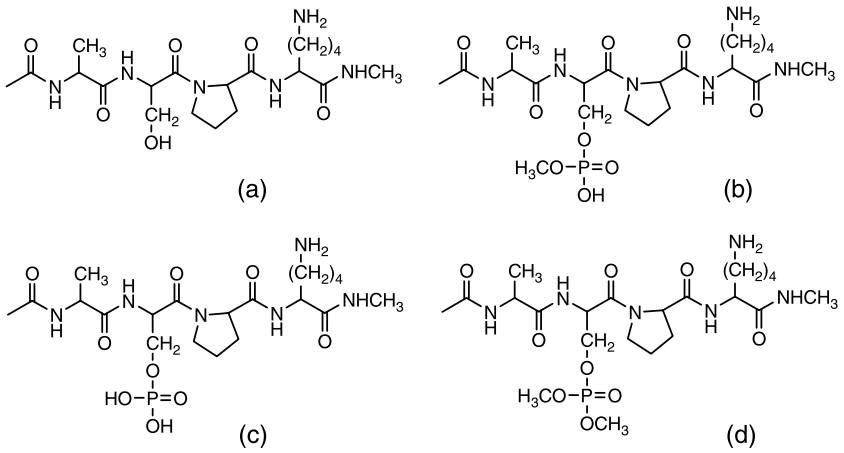

Figure 2. Chemical structure of designed tetrapeptides. At pH 4.7, without considering the positive charge induced by protonation of the lysine side-chain, the carried charge on these peptides should be (a) zero; (b) 1 negative charge; (c) nearly 1.5 negative charges; and (d) zero charge.

The C—N bond between proline and the other amino acid residue preceding proline was normally called the peptidyl-prolyl imide bond. The imide bond torsion, namely dihedral angle ω, was labeled in bold style, as shown in figure 1. The geometric isomers at the angles $\omega \approx 0^\circ$ (*cis*) and $\omega \approx \pm 180^\circ$ (*trans*), which were separated by a rotational barrier corresponding to the perpendicular high energy state of $\omega \approx 90^\circ$, were thought to be of comparable thermodynamic stability. The dihedral angle was not fixed, but fluctuated in a range of $\pm 20^\circ$.

3.2 Conformational searches

MD/SD searches were performed on the four designed tetrapeptides followed by multi-conformer minimization. The structures of eight located most stable configurations were shown in figure 3, and the peptidyl-prolyl imide bond dihedral angle and the lowest energy data were listed in table 1.

Proline residues show a relatively high intrinsic probability of having the *cis* rather than the *trans* isomer of the preceding peptide bond compared with other amino acids [28]. Energy calculations by Wuthrich suggested that the standard free energy ΔG° for the equilibrium was within the range of $4-8\,\mathrm{kJ\,mol^{-1}}$. The activation energy barrier for *cis*/*trans* isomerization of proline, $52\,\mathrm{kJ\,mol^{-1}}$, was also smaller compared with that of other regular peptide imide bonds, about $80\,\mathrm{kJ\,mol^{-1}}$ [29]. In our simulation the result was normally consistent with the above-mentioned law and the data summarized in table 1 revealed that the *cis* isomers were energetically less stable than *trans* isomers by $4-20\,\mathrm{kJ\,mol^{-1}}$. The dihedral angle fluctuated in a range of $\pm 20^\circ$ around the 0 and $\pm 180^\circ$.

3.3 Conformational analysis

We had originally postulated that the positively charged side-chain of Lys residue in model compounds would result in the formation of hydrogen bond and other charge–charge interaction. Whereas it is indicated that conformations of these four peptides were seemingly open structures and showed a very similar spatial arrangement (figure 3), in which the large side-chain of Lys was not close to any other groups at all. Although the bulky negative charged phosphate group and other electronegative groups, such as carbonyl at N-terminal,

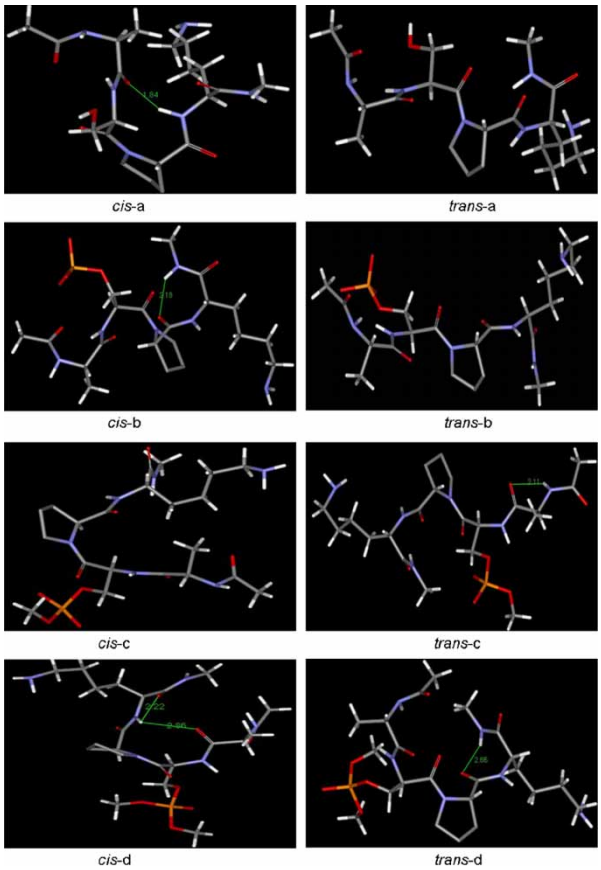

Figure 3. Structures of located most stable conformations. Hydrogen atoms except the αH on pyrrolidine ring of proline are not shown in order to retain clarity. Backbone and carbon atom are in gray color; oxygen red, phosphorus orange, hydrogen white, nitrogen blue. Hydrogen bonds are indicated by green lines. These figures were generated by use of Insight II and processed by WebLab ViewerLite software (colour in online version).

Table 1. The dihedral angle and energies for each conformation of model compounds shown in figure 2.

Entity	Dihedral angle ω (degree.)			The lowest energy (kJ mol^{-1})		
	cis	*Trans*†	*trans*‡	*cis*	*Trans*†	*trans*‡
a	6.07658	164.7387	− 170.0622	− 1064.8	− 1059.087	− 1084.486
b	− 34.57013	177.3437	− 157.9146	− 1316.444	− 1320.966	− 1312.54
c	− 13.68724	172.2216	− 175.5168	− 1322.42	− 1313.592	− 1330.917
d	19.84746	176.3892	− 172.469	− 1290.332	− 1300.612	− 1295.298

† Dihedral angle near the − 180°. ‡ Dihedral angle near the + 180°.

could theoretically bind to the positively charged side-chain of Lys, the stronger electrostatic repulsion induced between these large groups seems to prevent the actual occurrence, which results in remarkable side-chain diffused orientations. The NMR 2D-NOESY experiment of compound b (shown in figure 2b) also suggested an extensive structure. NOESY spectra in figure 4 provide information of short proton–proton distances in the range from the van der Waals distance of 2.0 Å to approximately 4.5 Å [30]. A few cross-peaks can be observed in figure 4, and the weak NOE signals prove that those protons of peptide are far away from each other, which indicates an random structure. The simulated interatomic distance (table 2) between Ala—CαH and Lys—CαH was found to be in the range of 5.46–6.39 Å, which is obviously larger than the required NOE interproton distance (2.0–4.5 Å). Thus the results of simulation are consistent with that found in the NMR spectra.

3.4 β-*Turn structure of cis*-**a** and *cis*-**d**

Although it was reported that proline-containing sequences of short linear peptides could have significant secondary structure in water [31–33], here these conformations did not show any hints other than *cis*-**a** and *cis*-**d**, in which the interproton distance were close to those of a β-turn and the turn was stabilized by a hydrogen bond (figure 3).

Cis imide bonds had a marked preference for turns and bends in polypeptide chain. The *cis* isomer is often favored in cyclic peptides and *cis* proline has been shown to occupy preferentially the $i + 2$ position of βVI turns. The choice between βVIa and βVIb turn was complicated by the fact that both turn ties were characterized by similar backbone angle ranges (BARs). The main difference between two BARs was the absence of hydrogen bond between the *i*-residue carbonyl oxygen and the $i + 3$ residue imide hydrogen in the βVI b turn [34]. The interatomic distance between the α carbon of the *i* and $i + 3$ residue has been defined to be between 4 and 7 Å [35] in a β-turn conformation and less than 3.10 Å for the intramolecular hydrogen bond formation. Thus, as shown in table 2, it is clear that *cis*-**a** and *cis*-**d** conformations, with the distance 5.46 and 6.39 Å between Ala—CαH and Lys—CαH, and 1.84 and 2.82 Å between Ala—(CO) and Lys—(NH), fit nicely the above-mentioned qualifications and thereby elucidate βVIa turn structures. Moreover, the distance between Ala—CαH and Lys—CαH for peptide

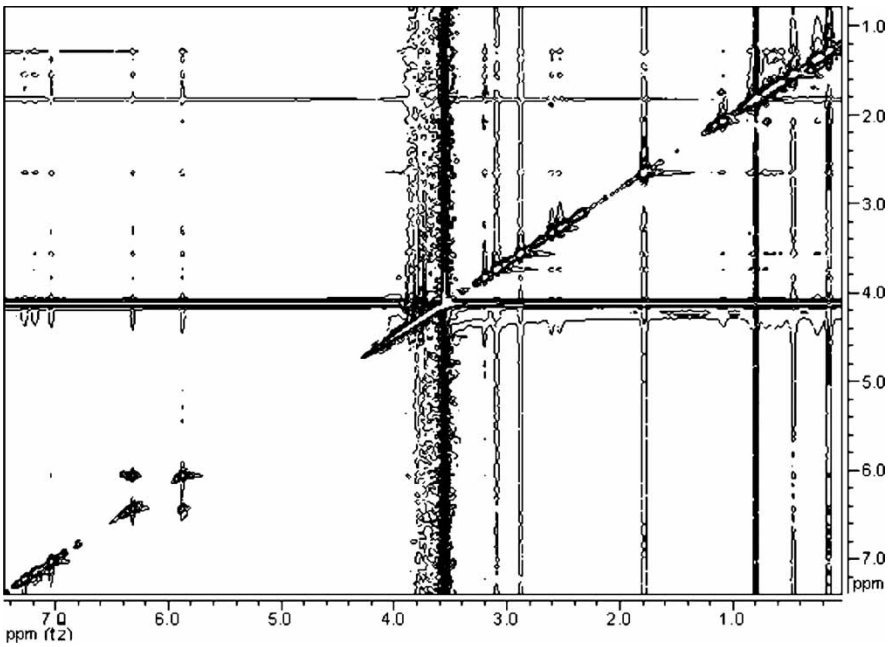

Figure 4. Total spectra of a 500 MHz NOESY of compound b in D_2O measured at 298 K.

Table 2. Interatomic distances (Å) between i and $i+3$ residue of the model peptides.

entity	Distance between Ala—CαH and Lys—CαH			Distance between Ala—(CO) and Lys –(NH)		
	cis	*Trans*†	*trans*‡	*cis*	*Trans*†	*trans*‡
a	5.46	10.15	9.97	1.84	5.89	5.89
b	8.47	10.01	9.73	5.78	6.56	6.08
c	6.82	6.07	10.14	5.38	6.82	6.82
d	6.39	9.92	9.50	2.82	6.39	6.68

† Dihedral angle near the −180°. ‡ Dihedral angle near the +180°.

cis-**c** (6.82 Å) also satisfies the precondition of a β turn. Since there was no hydrogen bond formed because of the 5.38 Å distance between Ala—(CO) and Lys—(NH), *cis*-**b** conformation exhibits a βVIb turn structure. These results indicated that the charge state of phosphate moiety affects the conformations of these peptides. The phosphorylated-peptide with more than one negative charge and having two sites for the negative charges is not helpful to a turn structure for peptide *cis*-**c**. Compared with the *cis*-**c**, the phosphorylated-peptide with only one negative charge has little impact on the configuration of the peptide *cis*-**b**. Therefore, it can be preliminarily concluded that the zero anionic state facilitates the formation of a regular conformation.

In fact, the turn structures observed in the *cis* conformations, the results of simulation in this paper, do not contradict the experimental result of 2D NOESY spectra shown in figure 4. The *cis* conformation signals were even almost invisible in 2D TOCSY in normal NMR conditions due to their very low concentration against the *trans* conformation fraction. Therefore, the conclusion of the random structures for these peptides deduced from the 2D NOESY is not unexpected as a whole.

3.5 Molecular dynamics/stochastic dynamics

In most free polypeptide chains, the occurrence of the *trans* form was more common, yet proline is the only amino acid in globular proteins with the highest probability of adopting a *cis* Imide bond. The *cis* and *trans* conformations of Xaa–Pro bonds have comparable energies, hence there are a 10–30% population of *cis* Xaa—Pro in the unfolded assemble and a ~6% frequency of *cis* Xaa—Pro bonds in native proteins [36]. In the case of our model peptides, by the MD/SD simulations, 100 low energetic conformations for each tetrapeptide were obtained and the distribution ratio was displayed in figure 5. The *cis* contents from the statistic results were 30, 19, 13 and 34% for the peptides **a**, **b**, **c** and **d**, respectively. The phosphorylated-peptide **d** with zero negative charge on its phosphate moiety got the most *cis* population among these four peptides, which was basically in good agreement with the results from our NMR experiments and the results reported by Schutkowski *et al.* [37]. In another model system Ac–Ala–Ser–Pro–Lys–NH–Np, the *cis* population calculated from ^{1}H NMR analysis were 7.0% for non-phosphorylated-peptide, 19.15, 22.0 and 29.15% for phosphorylated peptides corresponding to model compounds **c**, **b** and **d**, respectively (table 3). According to table 3, the results of unphosphorylated forms did not match well, which might be interpreted by the differences in model compounds. In model compound Ac–Ala–Ser–Pro–Lys–NH–Np, compounds were all

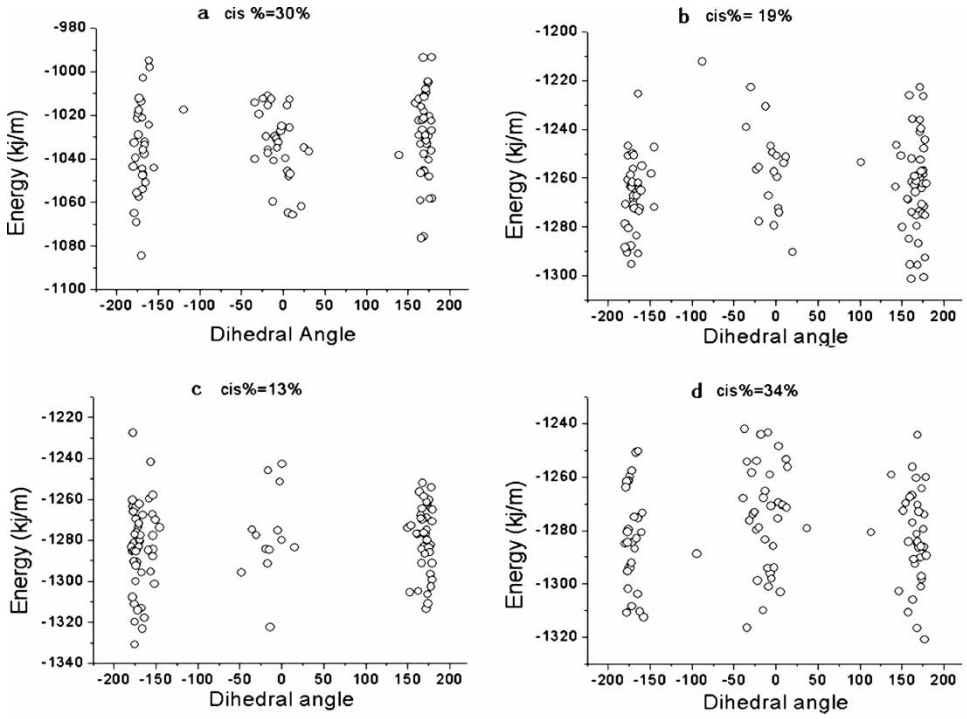

Figure 5. Low energetic conformation distribution of the four tetrapeptides.

Table 3. The contrast of the *cis* content (%) between simulated and experimental data.

	The cis content of unphosphorylated peptide	*The cis content of phosphorylated peptides*		
		d	***b***	***c***
Simulated data[†]	30	34	19	13
Exp. data[‡]	7	29.1	22.0	19.15

[†] Simulated in the model of Ac—Xaa—Pro—Lys—NH—Me. [‡] Detected in the model of Ac—Xaa—Pro—Lys—NH—Np.

modified with a large 4-nitroanilide group —NH—Np in the C terminal to facilitate the following thermodynamic research, nevertheless the model Ac–Ala–Ser–Pro–Lys–NH–Me were blocked with the smaller carboxyl-terminal blocking group —NH—Me to reduce the computational intensity. Brandts [28] suggested that adjacent residues, depending on their bulkiness, might influence the isomerization of proline residues. And Levitt also considered that the nature of the protein matrix in the areas of proline residues might greatly influence the conformation of the peptide bond. Therefore, the environmental diversities around the proline residue might be responsible for the difference.

In most cases, however, there are more or less differences between the results of experiments and simulations. Furthermore, the studied tetrapeptides may also be different, to some extent, with the corresponding units in true proteins. Therefore, some further experimental studies has been carrying out in our lab.

4. Conclusions

Based on the designed model Ac–Ala–Ser–Pro–Lys–NH–Me, the effect of phosphorylation on the peptidyl-prolyl imide bond *cis*/*trans* isomerization was studied by the molecular simulation method. The eight located most stable structures indicated that this kind of modification is highly related with the regulation of the peptides conformations. The more the negative charges are, the less the structures of those peptides are likely to form regular conformations, such as turns. Moreover, peptide with no negative charges on its phosphate moiety had the maximum *cis* isomer population, which also illuminates that the control of phosphorylation on the conformation of the peptides runs like a molecular switch.

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