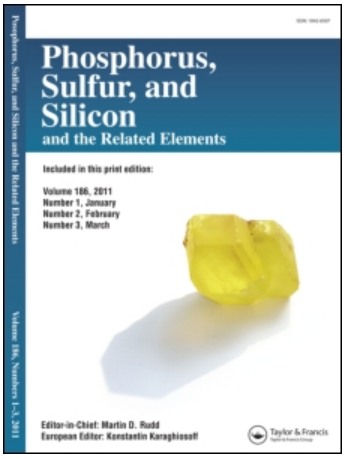

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Studies on the Reaction between Peptides or Proteins with N-Phosphoryl Amino Acids in Aqueous Solution

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Studies on the Reaction between Peptides or Proteins with N-Phosphoryl Amino Acids in Aqueous Solution

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In this article, it was found that N-phosphoryl α-amino acid could react with peptide or protein in aqueous solution to form the peptides or proteins increasing one acid amino residue. Through tandem-MS (MS/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS), it was found that the added amino acid residue was located at the N-terminal of material peptides or proteins. Furthermore, the added amino acid residue was from the N-phosphoryl α-amino acid. In this reaction process, a possible mechanism was proposed that five-membered ring penta-coordinate phosphorous intermediate played the vital role in the formation of peptide bond.

Keywords Five-membered ring penta-coordinate phosphorous intermediate; mass spectrometry; N-phosphoryl amino acids; peptide formation

INTRODUCTION

The origin of life on the earth is one of the most difficult scientific puzzles in life science.[1–3] More experiment results show that N-phosphoryl amino acids exhibit some biomimetic reactivity, such as

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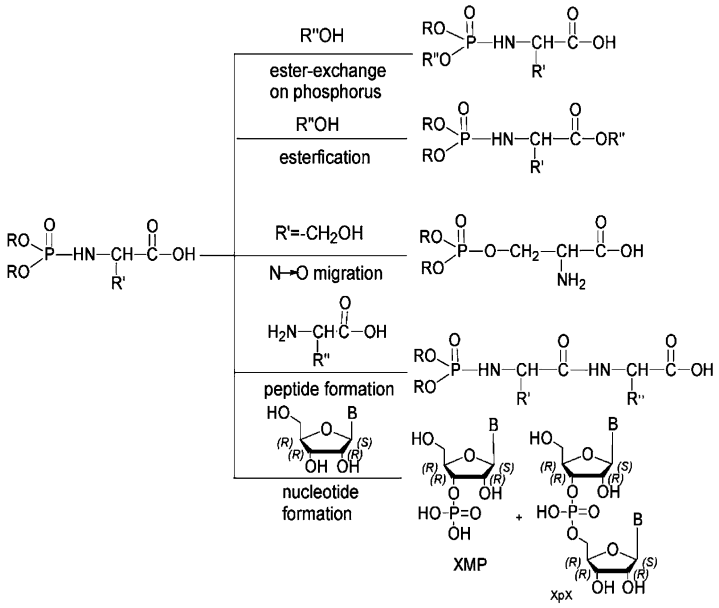

FIGURE 1 Chemical reactions of N-phosphoryl amino acids.

peptide formation,[4,5] ester formation,[6] ester exchange on phosphorus[7,8] phophonyl group N to O and N to S migration,[9–11] and oligonucleotide formation,[12] as shown in Figure 1. Based on the simple model, N-phosphoryl amino acid, a pathway of co-evolution of protein and nucleic acid was proposed.[13,14]

Previous studies on peptide formation reaction showed that N-phosphoryl amino acids could form short peptides after incubation in organic solution. If the reaction system changed to aqueous solution, only dipeptide and short poly-homohistidine peptide were obtained after incubation 90 days at 40°C.[15,16] To further investigate the features of the peptide formation reaction in aqueous solution, N-phosphoryl amino acids were incubated with peptides or protein (growth hormone releasing factor [GHRF] or cytochrome c) in 50 mM Tris·HCl at 37°C. The study on the corresponding chemical mechanism will be helpful to elucidate the basic principle of life.

RESULTS AND DISCUSSION

GHRF was incubated with N-(*O*, *O*′−diisopropyl)phospho-L-α-alanine (N-DIPP-L-α-Ala) for 24 h at 37°C in 50 mM Tris·HCl. After desalting by Zip tip$_{\mu-C18}$, the resulting mixture was analysed by electrospray ionization mass spectroscopy (ESI-MS). The deconvoluted

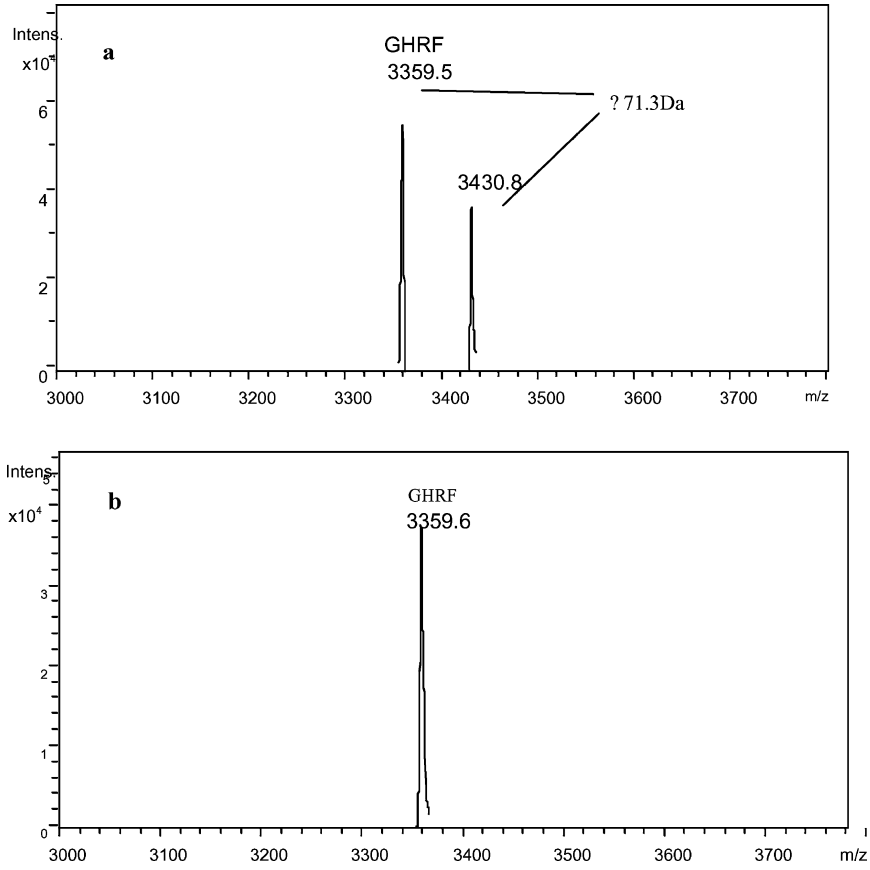

FIGURE 2 The deconvoluted mass spectram of GHRF incubation experiments. (a) GHRF incubates with N-DIPP-L-α-Ala, (b) GHRF in control group without N-DIPP-L-α-Ala.

mass spectrum (Figure 2a) shows two peaks, corresponding to the $[M_{GHRF} + H]^+$ ion at m/z 3359.5 and the ion at m/z 3430.8, respectively. The mass difference of the two ion peaks was 71.3 Da, corresponding to the mass number of alanine residue (Mw: 71.1). However, in control group without N-DIPP-L-α-Ala, only the $[M_{GHRF} + H]^+$ ion could be determined shown as Figure 2b. It was indicated that an alanine residue was capable of adding to GHRF.

Similarly, cytochrome c was incubated with N-DIPP-L-α-Ala for 6 days. After desalting and concentrateing by Millipore YM-3, the aqueous solution was analysed through HPLC-ESI-MS. The deconvoluted mass spectrum (Figure 3a) showed two peaks, $[M+H]^+$ at m/z 12364.1

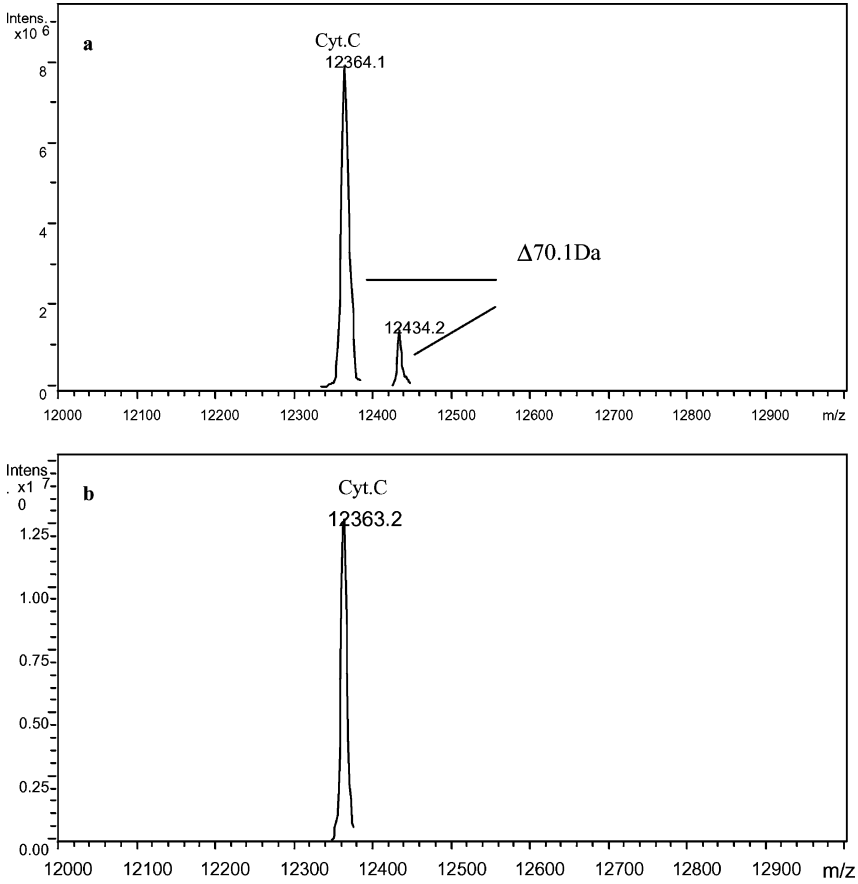

FIGURE 3 The deconvoluted mass spectram of cytochrome c incubation experiments. (a) cytochrome c incubation with N-DIPP-L-α-Ala. (b) cytochrome c in control group without N-DIPP-L-α-Ala.

and $[M+70.1+H]^+$ at m/z 12434.2, respectively, corresponding to molecular ion peak of Cytochrome C and a new peptide with addition of an alanine residue. The deconvoluted mass spectrum (Figure 3b) of control group showed only a $[M+H]^+$ion of Cytochrome C at m/z 12363.2.

Was the increment of the peptide bond length of the proteins due to the addition of alanine residue? Where the alanine residue came from? To answer these questions, alanylglutamine (Ala-Gln) was incubated with N-DIPP-α-Ala and with N-DIPP-α-Phe, respectively. Molecular weight of the mixtures was determined by ESI-MS. It was found that the $[M+H]^+$ ion at m/z 289 of Ala-Ala-Gln tripeptide was formed in the

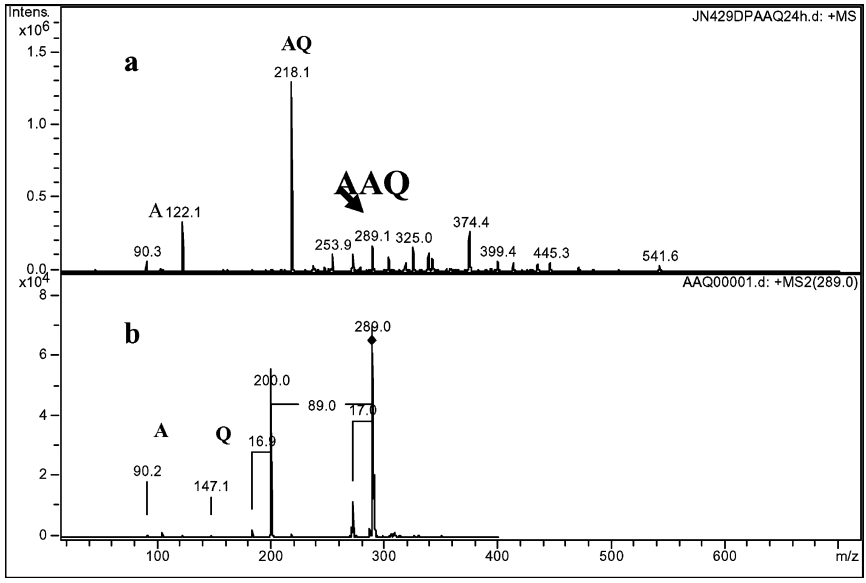

FIGURE 4 Mass spectra of Ala-Gln incubated with N-DIPP-α-Ala. (a) All mass of Ala-Gln incubated with N-DIPP-α-Ala; (b) MS2 spectra of $[Ala\text{-}Ala\text{-}Gln+H]^+$ ion at m/z 289.

incubation system containing N-DIPP-α-Ala (Figure 4), which was proved by secondary mass spectroscopy (MS2) spectram. In the same way, the $[M+H]^+$ ion at m/z 365 of Phe-Ala-Gln tripeptide was formed in the incubation system containing N-DIPP-α-Phe (Figure 5). The results showed that N-DIPP-α-Amino acid incubated with Ala-Gln could form the corresponding tripeptides, which could be described as X-Ala-Gln. And the N-terminal of the tripeptide was derived from N-DIPP-α-Amino acid. Consequently, to extend these model peptides to the proteins, it was concluded that the mass addition near 71.1Da mentioned above represented the alanine residue.

To elucidated the mechanism, the N-terminal blocked dipeptide Benzyloxycarbonyl-Ala-Gln (Z–Ala-Gln) was incubated with N-DIPP-α-Ala under the same condition. It was found that there was no tripeptide or other peptides containing Ala-Gln fragment could be identified by ESI-MS. The experiment result is implied that only the peptide containing free amino group could be involved in the peptide bond formation reaction.

Previous studies have reported that five membered ring pentacoordinate phosphorus intermediate was involved in peptides formation reaction in organic solution.[17,18] Accordingly, in this article, the

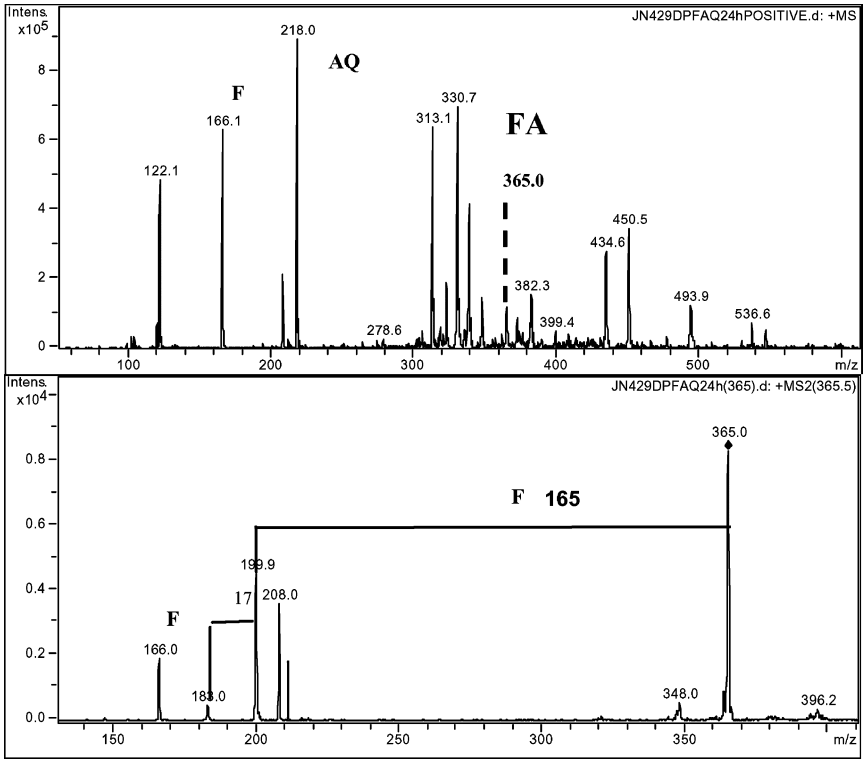

FIGURE 5 Mass spectra of Ala-Gln incubated with N-DIPP-α-Phe. (a) All mass of Ala-Gln incubated with N-DIPP-α-Phe;(b) MS2 spectra of $[M+H]^+$ ion of FAQ at m/z 356.

activated amino acid was speculated as a five membered cyclic pentacoordinate phosphoric-carboxylic mixed anhydride. The nucleophilic attack of the amino group of a peptide or protein on the carbonyl group of the intermediate led to the formation of peptide bond with the release of a phosphate ester (Figure 6).

In this article, N-DIPP-β-Ala and O-DIPP-α-Ser, were also chosen to be incubated with Ala-Gln in aqueous solution under the same condition, respectively. The mass spectra showed that there was no peek corresponding to any peptides formed even incubated for 17 days. Other forms of non-natural amino acids could not form peptides under similar condition as studied experimentally and theoretically by our laboratory previously.[19–21] This mechanism suggested that why nature chose α-amino acids as primary building blocks of protein.

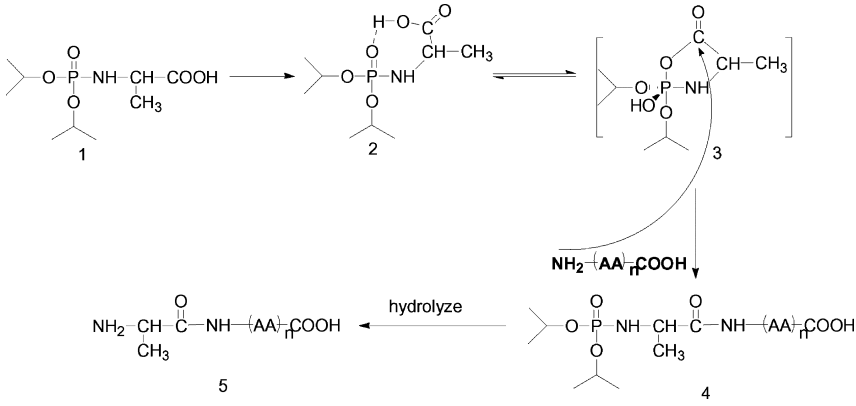

FIGURE 6 The mechanism of N-DIPP-α-Ala induced peptide bond formation reaction. 1. N-DIPP-α-Ala; 2 The five-membered ring penta-coordinate phosphoric intermediate; 3 attacking by N-terminal α-NH_2 group of peptides/proteins; 4. DIPP-peptides as the product of the reaction; and 5. Ala-peptide as the final product.

In conclusion, in this article, it was found that N-phosphoryl amino acids possess the ability to form peptides and proteins in aqueous solution under mild condition. Due to the interaction of N-phosphoryl α-amino acids such as N-DIPP-L-α-Ala with some different length peptides or proteins, such as GHRF (29 amino acid residues) and cytochrome c (104 amino acid residues), one amino acid residue was introduced to their N-terminal amino group respectively in aqueous solution. This study indicated that N-phosphoryl amino acids are good amino acid suppliers for proteins synthesis.

EXPERIMENTAL

Materials

Amino acids were from Baitai Biochemical Co. (Sigma agency). GHRF was from CL. Inc. (CL. XIAN Bio. Scientific Co. Ltd.). Cytochrome c was from Sigma (Chemical Co., USA). DIPPH, DMPH, Alanylglutamine (AQ), and Benzyloxycarbonyl-Alanylglutamine (Z-AQ) were synthesized in our laboratory. N-phosphoryl amino acids (N-DIPP-α-Ala, N-DIPP-α-Phe,) were prepared as described previously.[22,23] Buffer solution was 50 mM Tris·HCl water solution, pH 7.4, including 10mM Ca^{2+}.

Incubation

Two different length peptides and proteins, such as GHRF (29 amino acid residues), and cytochrome c (104 amino acid residues), were

selected to react with N-phosphoryl amino acids respectively. The resulting reaction solutions were analyzed by mass spectrometry.

N-(O, O′-diisopropyl)phospho-L-α-alanine (N-DIPP-L-α-Ala) (5 mg) and GHRF, cytochrome c (1 mg each) were dissolved and incubated in 1.5 ml buffer solution at 37°C for 24 h or 6 days. The corresponding control group experiments were carried out parallelly without N-DIPP-L-α-Ala. The resulting mixtures were desalted and concentrated using Zip $tip_{\mu-C18}$or YM-3 (Millipore, Bedford, MA), then were determined by electrospray ionization ion trap mass spectrometry (ESI-ion trap-MS) and LC-ESI-MS. The final acidity of incubation system was pH 3.5. The purpose of using buffer is to control the acidity of the reaction system. Excessive acidity may lead to high hydrolysis of N-phosphoryl amino acids in aqueous phase. Buffer free incubation system lead to fewer yields.

Alanylglutamine (AQ; 0.1 mmol) was reacted with N-DIPP-α-Ala, N-DIPP-α-Phe, at the same condition for 24 h or 17 days respectively, then, the resulting mixtures were determined by ESI-ion trap-MS.

N-NIPP-α-Ala (0.1 mmol) was incubated with Z-Alanylglutamine (Z-AQ) at the same condition for 24 h. The resulting mixture was determined by ESI-ion trap-MS.

ESI-MS Conditions

ESI-MS analysis was performed on Bruker Esquire 3000 plus (Brucker Dalton. Co., Germany) equipped with an electrospray ionization source. Operating conditions for ESI in the positive ion mode were as follow: Spray voltage, 4000 V; target, 350 m/z; capillary temperature, 300°C; dry gas (N_2), 7.5 L/min; Nebulizer (N_2), 4.5 psi. Mass spectra were registered in the scan range from 50 to 700 m/z.

LC-MS Conditions

To analyze incubated cytochrome c by LC-MS, an ion trap mass spectrometer (Bruker Esqiure 3000 plus, Brucker Dalton. Co., Germany) equipped with an electrospray ionization source was coupled to an Agilent 1100 binary pumping system (Agilent1100 Technologies, Wilmington, DE) and an ultraviolet (UV) detector. HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were purchased from TEDIA COMPANY.INC. The HPLC column was ZORBA × 300SB C18, 250 × 2.1 mm (Agilent, Co. USA). The column was thermostated at 25°C. The mobile phase consisted of 0.02% TFA in 90% water and 10% acetonitrile (Solvent A) and 0.02% TFA in 90% acetonitrile and 10% water (Solvent B). The gradient program with a flow rate of 0.3 mL/min from 0% to 20%

Solvent B in 5 minutes, then from 20% to 100% Solvent B in 55 minutes, was applied for the LC-MS analysis of the sample. The UV detection was performed at 220 nm. LC-MS experiments were performed using an ion trap (Bruker ESQUIRE 3000 Plus) mass spectrometer equipped with an ESI ion source. Operating conditions for ESI in the positive ion mode were as follow: Spray voltage, 4000 V; target, 1500 m/z; capillary temperature, 365°C; dry gas (N_2), 10 L/min; Nebulizer (N_2), 35 psi. Mass spectra were registered in the scan range from 400 to 3000 m/z. About twenty percent of the effluent from UV absorbance detector was introduced to the electrospray mass spectrometer through a splitting T valve. The ESI-MS spectra were calibrated externally with standard mixture obtained from Agilent.

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