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Amino Acids

α-Amino acid behaves differently from β- or γ-amino acids as treated by trimetaphosphate

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Summary. The condensation reactions of sodium trimetaphosphate with single amino acids, namely glycine, L-alanine, β-alanine and γ-aminobutyric acid or pairs of these amino acids were reinvestigated by electrospray ion-trap mass spectrometry and high performance liquid chromatography. It was found when mixtures were treated by sodium trimetaphosphate only in the presence of α-amino acid dipeptides were formed. Without addition of α-amino acids, the β-amino acid or γ-aminobutyric acid could not form peptide either by themselves or with their mixtures under the same conditions. From the data it is concluded that phosphate might select α-amino acids to produce the peptides being important precursors for the origin of life.

Keywords: Trimetaphosphate – α-Amino acids – β-Amino acids – γ-Amino acids – Peptide – LC-ESI-MS

1. Introduction

It was suggested that probably inorganic condensed phosphates, such as polyphosphate and trimetaphosphate, would have been the essential materials for the prebiotic evolution which preceded the emergence of life on the earth. Rabinowitz et al. (1969, 1970) demonstrated that some amino acids such as glycine and L-alanine could be condensed to dipeptides in aqueous solutions by the treatment of trimetaphosphate at room temperature. Chung et al. (1971) suggested that the mechanism of the trimetaphosphate-induced peptide synthesis reaction might involve five-membered active cyclic phosphate intermediates. However, most of the phosphorus on the earth would have been in the form of water-insoluble apatite, so it is hard to understand the prebiotic origin of trimetaphosphate in nature. Yamagata et al. (1991) found that volcanic activity could produce water-soluble polyphosphate including a small amount of trimetaphosphate which was also detected from the experiments simulating magmatic conditions. Tsuhako et al. (1985) and Inoue et al. (1993) investigated the reaction of amino acids with trimetaphosphate and obtained the direct proof of the five-membered cyclic anhydride by ^{31}P NMR. They also found that glycine and alanine reacted with sodium trimetaphosphate to yield the homo-dipeptides, whereas the formation of dipeptides was not observed in the reaction of valine, serine and β-alanine with trimetaphosphate. On the other hand, sodium trimetaphosphate has also been used as the reagent for the phosphorylation of nucleoside (Tsuhako et al., 1984; Cheng et al., 2002; De Graaf and Schwartz, 2005). Recently, this reaction was used to produce longer oligopeptides at different reaction conditions (Yamagata and Inomata, 1997; Hill and Orgel, 2002).

In recent years, alternative methods for the analysis of amino acids and small peptides without derivatization have been proposed. Ion-pair chromatography and reversed-phase liquid chromatography were widely used to separate amino acids and small peptides by employing perfluorinated carboxylic acids such as trifluoroacetic acid (TFA), pentfluoropropionic acid (PFPA) and nonafluoropentonoic acid (NFPA) as ion-pairing reagents on a silica based column (Petritis et al., 1999). Furthermore, electrospray tandem mass spectrometry (ESI-MS) was an effective direct detector for analysis of underivatized amino acids and peptides due to its high sensitivity and incom-

parable specificity (Chaimbault et al., 2000; Petritis et al., 2002; Person et al., 2004). In this work, on-line ion-pair reversed-phase liquid chromatography (IP-RPLC) and ESI-MS were used to investegate the peptides which were formed by reaction of amino acids with sodium trimetaphosphate under reasonably plausible prebiological conditions (slightly basic, low temperature and low concentration).

2. Materials and methods

2.1 Chemicals

Glycine (Gly), L-alanine (Ala), β-alanine (β-Ala) and γ-aminobutyric acid (γ-Aba) were purchased from GL Biochem Ltd. (Shanghai, China). Nonafluoropentonoic acid (NFPA) and sodium trimetaphosphate (P_{3m}) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). HPLC grade acetonitrile was purchased from Tedia Company Inc. (Fairfield, OH, U.S.A.). Strong acidic cation-exchange resin (Diaion SK1B) was purchased from H&E Co. Ltd. (Beijing, China). Unless specified otherwise, all chemicals and solvents were analytical reagents. Authentic dipeptide of Gly–Ala was synthesized according to the published method (Yang et al., 2003). Structure and purity of Gly–Ala were checked by HPLC and NMR. ^{1}H NMR (400 MHz, D_2O): $\delta = 1.26$ (d, $J = 7.2$ Hz, 3H, CH_3), 3.73 (s, 2H, CH_2), 4.07 (dd, $J = 7.2$ Hz, 1H, CH) ppm. ^{13}C NMR (100 MHz, D_2O): $\delta = 17.09$, 40.41, 51.13, 166.14, 179.95 ppm. MS (ESI): $m/z = 147$ ($M + H^+$, 100%), 169 ($M + Na^+$, 7%).

2.2 Reaction of amino acids with sodium trimetaphosphate

A 20 mL aqueous solution of each amino acid (0.1 M) namely glycine, L-alanine, β-alanine or γ-aminobutyric acid and P_{3m} (0.1 M) was prepared. Experiment (a): glycine (0.1 M), L-alanine (0.1 M) and P_{3m} (0.2 M); experiment (b): glycine (0.1 M), β-alanine (0.1 M) and P_{3m} (0.2 M); experiment (c): glycine (0.1 M), γ-aminobutyric acid (0.1 M) and P_{3m} (0.2 M); experiment (d): β-alanine (0.1 M), γ-aminobutyric acid (0.1 M) and P_{3m} (0.2 M).

These solutions in flasks were then adjusted to the predetermined pH 10 by the addition of 2 M NaOH, and stirred at room temperature under argon atmosphere for 7 or 8 days. The pH of the solutions was always kept at about 10 during the process of the reactions. To the reaction media small amounts of toluene are added as antiseptics. After 7 days, the pH of the solutions were adjusted to about 6 with 1 M HCl. Then the solutions were de-salted by cation-exchange resin in order to remove the phosphates which were harmful to mass spectrometer. The general desalting procedures were as follow. Glass column (20 mm i.d. and about 15 cm long) was fitted with a plug of glass wool. A suspension of Diaion SK1B resin (H^+-form, 50 g) was poured into the column. The column was washed with 50 mL 1 M HCl and then with 100 mL water. To the column 15 mL of reaction solution was added slowly, and then the column was washed with about 50 mL water to remove of the phosphates. About 60 mL 3% ammonia were run through the column (1 drop/sec) and the eluant traced by ninhydrin reaction was collected in tubes. After water was removed by evaporation in vacuo, the remaining white solid were checked by ESI-MS and LC-MS.

2.3 Mass spectrometric conditons

Mass spectra were acquired using a Bruker Dalton Esquire 3000 plus ion-trap mass spectrometer (Bruker-Daltonik Co., Bremen, Germany) equipped with an electrospray ionization source, capable of analyzing ions up to $m/z = 6000$. The mass spectrometer was operated in the positive ion mode. Nitrogen was used as the dry gas at a flow-rate of $4\,L \cdot min^{-1}$. The nebulizer pressure was 4.83×10^4 Pa and the electrospray needle was typically held at 4000 V. The heated capillary temperature was 250 °C. The samples dissolved in methanol-water (1:1) were ionized by ESI and continuously infused into the ESI chamber at a flow-rate of $0.24\,mL \cdot h^{-1}$ by a Cole-parmer 74900 syringe pump (Cole-Parmer Instrument Co., Vernon Hills, IL, U.S.A.). Five scans were averaged for each spectrum.

2.4 HPLC-MS conditions

In order to analyse the reaction samples by LC-MS, an ion trap mass spectrometer described above was coupled to an Agilent 1100 binary pumping system and an UV detector (Agilent 1100 technologies, Wilmington, DE, U.S.A.). Chromatography column: Supelcosil ABZ + plus 150 × 4.6 mm i.d. (Supelco, Bellefonte, PA, U.S.A.). The column was thermostated at 25 °C. Solvent A: 2 mM NFPA in water; solvent B: acetonitrile. The eluant program with a flow-rate of $1.0\,mL \cdot min^{-1}$ at 4% B in 10 min, was applied for the LC-MS analysis of the samples. The injection volume was 20 μL. The UV detection was performed at 220 nm. Operating conditions for ESI in the positive ion mode were as following: Spray voltage: 4000 V; target: $m/z = 133$; capillary temperature: 300 °C; dry gas (N_2): $10\,L \cdot min^{-1}$; nebulizer (N_2): 2.06×10^5 Pa. Mass spectra were registered in the scan range from $m/z = 50$ to 300. For LC-ESI-MS, about 1/10 of

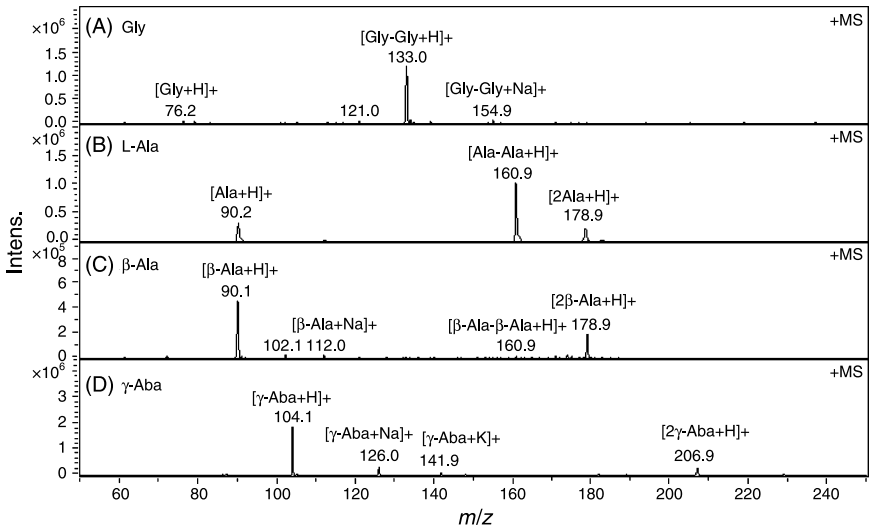


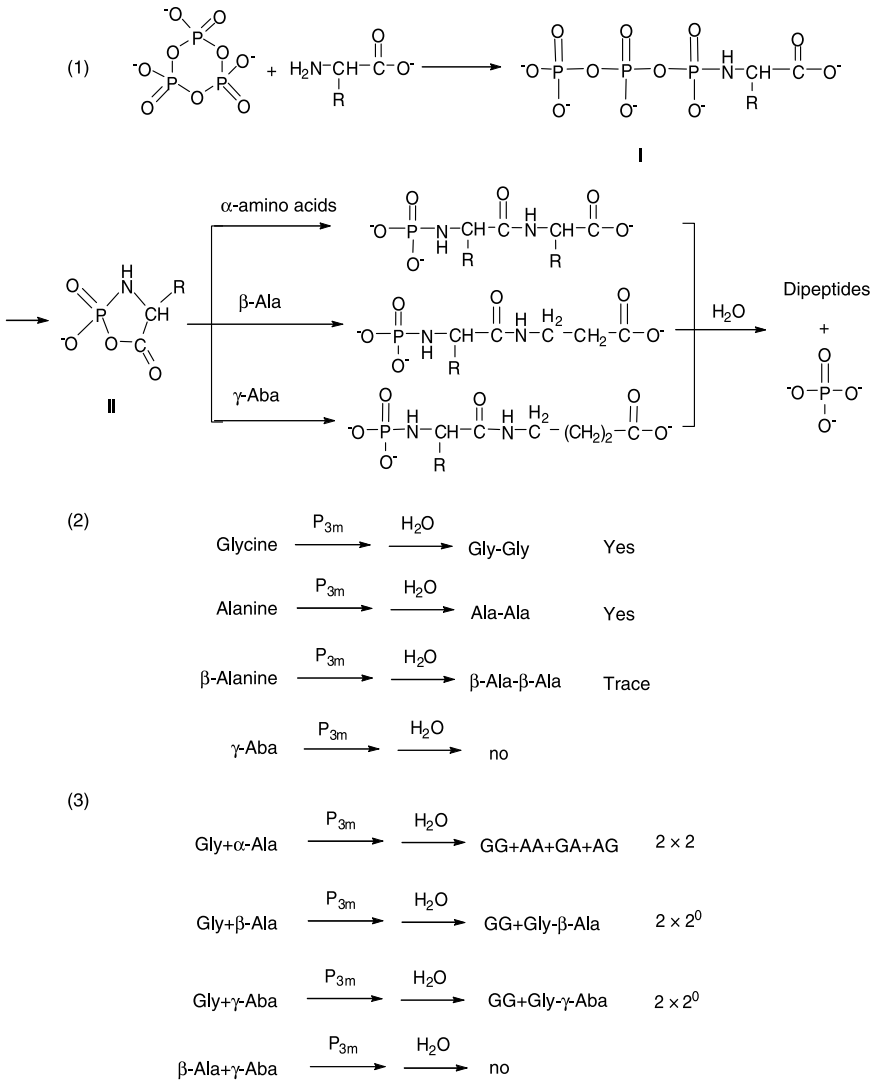
Fig. 1. The ESI-MS spectra for each amino acid treated with P_{3m}. (**A**) Glycine reacted with P_{3m}. (**B**) L-Alanine reacted with P_{3m}. (**C**) β-Alanine reacted with P_{3m}. (**D**) γ-Aminobutyric acid reacted with P_{3m}

the effluent from UV absorbance detector was introduced to the electrospray through a splitting T valve in order to avoid a too high flow-rate in the ion source.

3. Results and discussion

When single amino acids namely glycine, α-alanine, β-alanine or γ-aminobutyric acid were treated with P_{3m}, respectively, it was found that glycine (corresponding to Fig. 1A) and L-alanine (corresponding to Fig. 1B) could easily form dipeptides Gly–Gly providing $m/z = 133$ and Ala–Ala providing $m/z = 161$, respectively, but β-alanine (corresponding to Fig. 1C) could only form trace amount of β-Ala–β-Ala providing $m/z = 161$ as determined by ESI-MS^2. Furthermore, γ-aminobutyric acid (corresponding to Fig. 1D) was almost impossible to produce any peptide under the same conditions. Based on this experiment, it seems that when treated by P_{3m} the order for the extent of peptide formation is α≫β≫γ amino acids (Scheme 1(2)).

However, how about the mixed amino acids samples, such as glycine was mixed with α-alanine, β-alanine or γ-aminobutyric acid, respectively? From Fig. 2A, for the case of glycine and L-alanine mixture, it was found that there were five mass peaks at $m/z = 76$, 90, 133, 147 and 161, corresponding to Gly, L-Ala, Gly–Gly, Gly–Ala (or Ala–Gly) and Ala–Ala, respectively. In other words, four dipeptides including two homo- and two hetero-dipeptides were formed. On the contrary, the ESI-MS data of experiments (b) and (c) in Fig. 2B, C and Table 1 showed that there were only two dipeptides were formed, respectively. It is worth noting that when the mixture of β-alanine and γ-aminobutyric acid was treated by P_{3m} no peptide was formed (Fig. 2D).



Scheme 1. A possible mechanism of peptide formation in the reaction of trimetaphosphate with amino acids

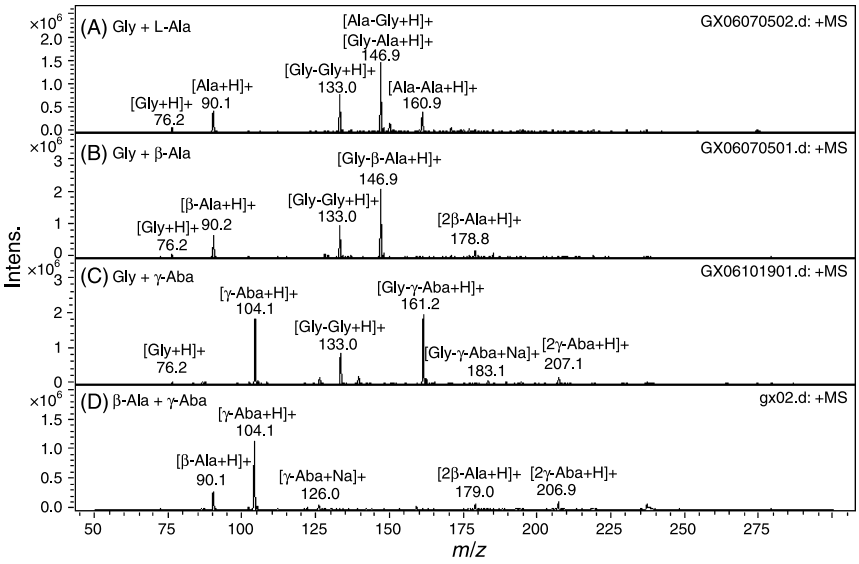


Fig. 2. The ESI-MS spectra of amino acid pairs treated with P_{3m}. (**A**) Glycine and L-alanine reacted with P_{3m}. (**B**) Glycine and β-alanine reacted with P_{3m}. (**C**) Glycine and γ-aminobutyric acid reacted with P_{3m}. (**D**) β-Alanine and γ-aminobutyric acid reacted with P_{3m}

Table 1. ESI-MS data for the products of four experiments (a)–(d)

Experiments	*m/z* (relative abundance, %)							
	Gly	Ala	β-Ala	Aba[1]	Gly–Gly	Gly-X[2]	X-Gly	X–X
(a)	76 (6)	90 (30)	–	–	133 (54)	147 (m_1)[3]	147 (m_2)	161 (29)
(b)	76 (4)	–	90 (34)	–	133 (46)	147 (100)	–	–
(c)	76 (3)	–	–	104 (94)	133 (44)	161 (100)	–	–
(d)	–	–	90 (29)	104 (100)	–	–	–	–

[1] *Aba*: γ-aminobutyric acid; [2] X = (a) Ala; (b) β-Ala; (c) γ-Aba; [3] $m_1 + m_2 = 100$

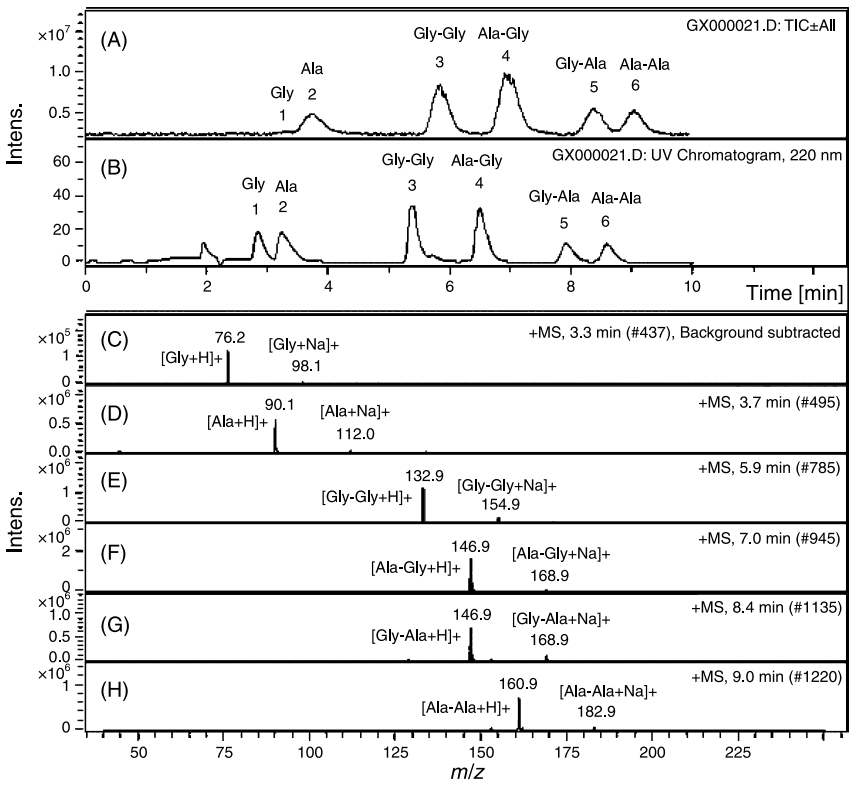


Fig. 3. LC-ESI-MS spectra of experiment (a) glycine and L-alanine treated with P_{3m}. (**A**) Total ion current (TIC) chromatogram. (**B**) Ultraviolet (UV) chromatogram, detection at 220 nm. (**C**) Peak 1, Gly. (**D**) Peak 2, L-Ala. (**E**) Peak 3, Gly–Gly. (**F**) Peak 4, Ala–Gly. (**G**) Peak 5, Gly–Ala. (**H**) Peak 6, Ala–Ala

The ESI-MS^n as a powerful technique for the determination of the amino acid identities and sequence of the protonated peptides were applied to explore the structures of these dipeptides (Biemann and Scoble, 1987; Papayannopoulos 1995). Also over the past decade, the fragmentation pathways and fragment-ion structures for the peptides have been elucidated to help understanding the tandem spectra (Cordero et al., 1993; Chen et al., 2000; Harrison et al., 2000; Paizs et al., 2004; Bao et al., 2005; Zhou et al., 2005). For example, the fragmentation reaction of $[Ala–Ala+H]^+$ ion at $m/z=161$ involve elimination of H_2O, followed by the loss of CO to provide the ion at $m/z=115$. The amide bond of the protonated peptide could cleave to produce the a_1 and y_1 ions.

Hence, these mixtures of experiments (a), (b) and (c) were efficiently separated by high performance liquid chromatography and investigated by LC-MS. Both the UV chromatograms and total ion current (TIC) chromatagrams showed that there were six peaks produced when glycine was mixed with α-alanine (Fig. 3A, B). LC-MS (Fig. 3C–H) gave the corresponding mass of each peak, which was assigned to the structure. To analyse these products quantitatively, all peaks were evaluated by their retention time as well as the peak areas (Table 2). It is shown that glycine was eluted at 2.7 min (peak 1) and Ala, Gly–Gly, Ala–Gly, Gly–Ala, and Ala–Ala at 3.1 min (peak 2), 5.5 min (peak 3), 6.5 min (peak 4), 7.9 min (peak 5) and 8.6 min (peak 6) respectively. Ala–Gly and Gly–Ala have the same molecular weight, so they can not be distinguished easily. To differentiate them, the authentic sample Gly–Ala was added into the reaction product, which caused the area of peak 5 to increase. Hence, peak 5 corresponds to Gly–Ala and peak 4 should be Ala–Gly. From the UV chromatograms, it can be deduced that the relative integration area of total dipeptides of experiments (a), (b) and (c) was 71%, 57% and 55%, respectively. From the data

Table 2. The HPLC data of compounds resulting from experiments (a)–(c)

Experiments	Peak numbers						Σ peptides (%)
	1	2	3	4	5	6	
(a)	Gly (12)[1]	Ala (17)	Gly–Gly (24)	Ala–Gly (26)	Gly–Ala (10)	Ala–Ala (11)	71
(b)	Gly (7)	Gly–Gly (26)	β-Ala (36)	Gly-β-Ala (31)	–	–	57
(c)	Gly (7)	Gly–Gly (25)	Aba (38)	Gly–Aba (30)	–	–	55

[1] The data in the parentheses is the relative integration area (%) of HPLC analysis

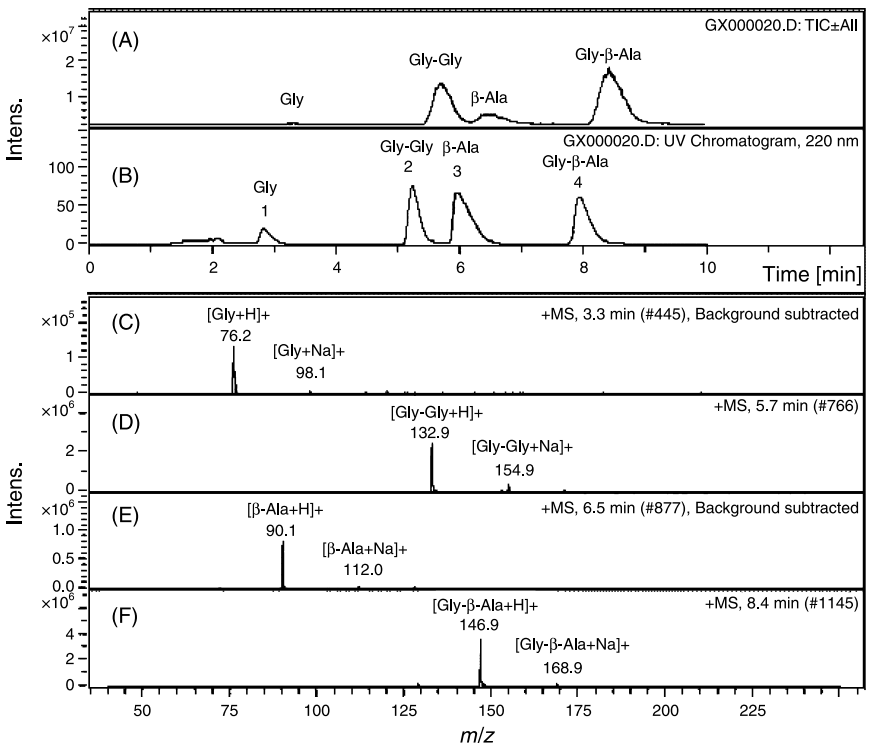


Fig. 4. LC-ESI-MS spectra of experiment (b) glycine and β-alanine treated with P_{3m}. (**A**) Total ion current (TIC) chromatogram. (**B**) Ultraviolet (UV) chromatogram, detection at 220 nm. (**C**) Peak 1, Gly. (**D**) Peak 2, Gly–Gly. (**E**) Peak 3, β-Ala. (**F**) Peak 4, Gly-β-Ala

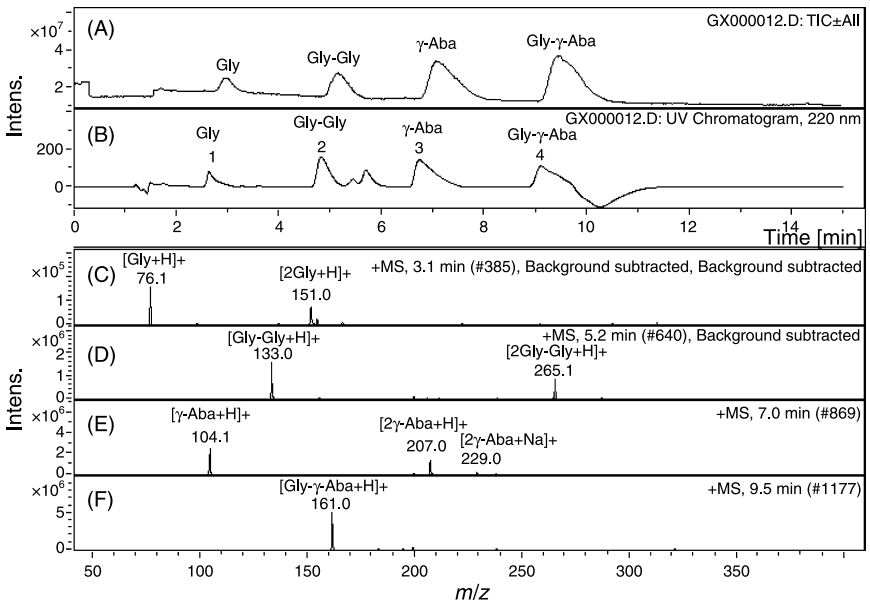


Fig. 5. LC-ESI-MS spectra of experiment (c) glycine and γ-aminobutyric acid treated with P_{3m}. (**A**) Total ion current (TIC) chromatogram. (**B**) Ultraviolet (UV) chromatogram, detection at 220 nm. (**C**) Peak 1, Gly. (**D**) Peak 2, Gly–Gly. (**E**) Peak 3, γ-Aba. (**F**) Peak 4, Gly-γ-Aba

it is concluded that the yield of peptide formation is (a) > (b) > (c). But when glycine was mixed with β-alanine or γ-aminobutyric acid, there were only four peaks formed as can be seen in Figs. 4 and 5, repectively. Their structures were also identified by the LC-MS as described above. It should be pointed out that, in the chromatogram shown in Fig. 5B, two unknown peaks emerge between peaks 2 and 3 which did not appear in the total ion current (TIC) chromatogram numbered in Fig. 5A. Therefore, it is assumed that the unknown ones were not ionizable compounds.

To understand the mechanism of the chemical reaction, a possible route is shown in Scheme 1. The first step is that the amino group of α-amino acids, β-alanine or γ-aminobutyric acid nucleophilically attacks a phosphorus atom on trimetaphosphate to form a triphosphorylated amino acid. But only for the α-amino acids, an intramolecular attack of the carboxyl group on a phosporus atom of triphosphorylated amino acids could yield a five-membered cyclic phosphate II as the crucial intermediate. The next step is that the amino group of another amino acid, such as glycine, alanine, β-alanine or γ-aminobutyric acid couple to the cyclic phosphate II to yield the monophosphorylated dipeptides, which, in turn, are easily hydrolyzed to dipeptides and phosphates. Thus, when mixture of glycine and L-alanine were treated with sodium trimetaphosphate, four dipeptides (2×2) Gly–Gly, Ala–Ala, Gly–Ala and Ala–Ala were formed (Scheme 1 (3)). However, for a β-amino acid or γ-aminobutyric acid it is very difficult to form a six- or seven-membered cyclic mixed anhydride. Consequently, there were almost no peptide formed either by themselves or with their mixtures. Only if they are mixed with the α-amino acid glycine peptides were formed. In other words, the presence of α-amino acids is important to form the dipeptides, and the phosphate catalyzes the peptide formation.

In conclusion, the treatment of P_{3m} with α-amino acid is different in comparison with β- or γ-amino acids. This might be the reason why phosphate choose α-amino acids as targets but not β- or γ-amino acids for the formation of proteins.

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References

Bao JY, Ai HW, Fu H, Jiang YY, Zhao YF (2005) Peptide sequencing through N-terminal phosphorylation and electrospray ionization mass spectrometry. J Mass Spectrom 40: 772–776

Biemann K, Scoble HA (1987) Characterization by tandem mass spectrometry of structural modifications in proteins. Science 237: 992–998

Chaimbault P, Petritis K, Elfakir C, Dreux M (2000) Ion-pair chromatography on a porus graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids. J Chromatagr A 870: 245–254

Chen J, Chen Y, Gong P, Jiang Y, Li YM, Zhao YF (2002) A novel phosphoryl derivatization method for peptide sequencing by electrospray ionization spectrometry. Rapid Comm Mass Spectrom 16: 325–329

Cheng CM, Fan C, Wan R, Tong CY, Miao ZW, Chen J, Zhao YF (2002) Phosphorylation of adenosine with trimetaphosphate under simulated prebiotic conditions. Orig Life Evol B 32: 219–224

Chung N, Lohrmann R, Orgel LE, Rabinowitz J (1971) The mechanism of the trimetaphosphate-induced peptide synthesis. Tetrahedron 27: 1205–1210

Cordero MM, Houser JJ, Wesdemiotis C (1993) The neutral products formed during backbone fragmentations of protonated peptides in tandem mass spectrometry. Anal Chem 65: 1594–1601

De Graaf RM, Schwartz AW (2005) Thermal synthesis of nucleosides H-phosphonates under mild conditions. Orig Life Evol B 35: 1–10

Harrison AG, Csizmadia IG, Tang TH, Tu YP (2000) Reaction competition in the fragmentation of protonated dipeptides. J Mass Spectrom 35: 683–688

Hill A, Ogel LE (2002) Trimetaphosphate-induced addition of aspartic acid to oligo (glutamic acid)s. Helv Chim Acta 85: 4244–4254

Inoue H, Baba Y, Furukawa T, Maeda Y, Tsuhako M (1993) Formation of dipeptide in the reaction of amino acids with *cyclo*-triphosphate. Chem Pharm Bull 41: 1895–1899

Paizs B, Schnolzer M, Warnken U, Shhai S, Harrison AG (2004) Cleavage of the amide bond of the protonated dipeptides. Phys Chem Chem Phys 6: 2691–2699

Papayannopoulos IA (1995) The interpretation of collision-induced dissociation tandem mass spectra of peptides. Mass Spectrom Rev 14: 49–73

Person MD, Sevestre A, Chaimbault P, Perrot L, Duchiron F, Elfakir C (2004) Characterization of low-molecular weight peptides in champagne wine by liquid chromatography/tandem mass spectrometry. Anal Chim Acta 520: 149–158

Petritis K, Brussaux S, Guenu S, Elfakir C, Dreux M (2002) Ion-pair reversed-phase liquid chromatography-electrospray mass spectrometry for the analysis of underivatized small peptides. J Chromatogr A 957: 173–185

Petritis K, Chaimbault P, Elfakir C, Dreux M (1999) Determination of 20 underivatized proteinic amino acids by ion-pairing chromatography and pneumatically assisted electrospray mass spectrometry. J Chromatagr A 855: 191–202

Rabinowitz J (1970) Peptide and amide bond formation in aqueous solutions of cyclic or linear polyphosphates as a possible prebiotic process. Helv Chim Acta 53: 1350–1355

Rabinowitz J, Flores J, Krebsbach R, Rogers G (1969) Peptide formation in the presence of linear or cyclic polyphosphates. Nature 224: 795–796

Tsuhako M, Fujimoto M, Ohashi S, Nariai H, Motooka I (1984) Phosphorylation of nucleosides with sodium *cyclo*-triphosphate. Bull Chem Soc Jpn 57: 3274–3280

Tsuhako M, Nakajima A, Miyajima T, Ohashi S, Nariai H, Motooka I (1985) The reaction of *cyclo*-triphosphate with L-α- or β-alanine. Bull Chem Soc Jpn 41: 3092–3098

Yamagata Y, Inomata K (1997) Condensation of glycylglycine to oligoglycines with trimetaphosphate in aqueous solution. Orig Life Evol B 27: 339–344

Yamagata Y, Watanabe H, Saitoh M, Namba T (1991) Volcanic production of polyphosphates and its relevance to prebiotic evolution. Nature 352: 516–519

Yang DC, Fan L, Zhong YG (2003) Synthesis of protected RGD tripeptide. Chin J Org Chem 23: 493–498

Zhou GJ, Liu Z, Wang TJ, Xu PX, Tang G, Ye Y, Zhao YF (2005) Electrospray ionization mass spectrometry study on dipeptide 4-chlorobutyl ester produced from refluxing of amino acids with phosphoryl chloride in tetrahydrofuran. J Mass Spectrom 40: 1382–1386

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