Formation of Dipeptide in the Reaction of Amino Acids with *cyclo*-Triphosphate

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The reaction of glycine (Gly) or L-α-alanine (Ala) with inorganic sodium *cyclo*-triphosphate hexahydrate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$ gave dipeptides and the phosphorylated products of amino acids, but the reaction of L-valine (Val) or L-serine (Ser) with P_{3m} showed no peptide formation. The phosphorylated products of these reactions have a P–N bond in their molecules. Gly reacted with P_{3m} to give *N*-(carboxymethyl)phosphoramidate (P_1-(N)Gly: 1a), 5-oxo-1,3,2-oxazaphospholidin-2-olate 2-oxide (P_1-(N,O)Gly: 2a), which was a five-membered cyclic anhydride, *N*-(*N*-phosphonoglycyl)glycine (P_1-N(GlyGly): 3a), *N*-(carboxymethyl)triphosphoramidate (P_3-(N)Gly: 4a), and glycylglycine (GlyGly). A five-membered cyclic anhydride (2a) which was formed *via* an intramolecular cyclization of 4a was found to be a key intermediate for the production of GlyGly. Phosphorylation of Ala with P_{3m} also gave alanylalanine (AlaAla) in addition to the phosphorylated products including a five-membered cyclic anhydride. The yields of GlyGly and AlaAla were 15.8 and 2.0%, respectively. In the reactions of Val and Ser with P_{3m}, only *N*-(1-carboxy-2-methylpropyl)phosphoramidate (P_1-(N)Val: 1c) and *N*-(1-carboxy-2-hydroxyethyl)phosphoramidate (P_1-(N)Ser: 1d) were formed and their dipeptides were not obtained at all. The mechanism of the dipeptide formation will be discussed.

Keywords dipeptide; amino acid; condensation; *cyclo*-triphosphate; phosphorylation

Rabinowitz *et al.*[1-3] and Feldmann[4] demonstrated 20 years ago that treatments of some amino acids such as glycine and L-α-alanine with *cyclo*-triphosphate (P_{3m}) afforded good yields of dipeptides under reasonably plausible prebiological conditions (slightly basic pH, low temperature, and low concentration). Several authors[5-8] have tried to extend this reaction to the formation of other dipeptides. More recently, Yamagata *et al.*[9] found volcanic production of polyphosphates, including *cyclo*-triphosphate (P_{3m}), and its relevance to prebiotic evolution. Much attention, therefore, has been concentrated on P_{3m} as a prebiotic condensing agent of bio-organic compounds. Prebiotic condensing agents[10] such as cyanamide, carbodiimide, dicyandiamide, and linear polyphosphates can advance dehydration condensation reactions of amino acids, nucleosides, and nucleotides, which are very unlikely to happen spontaneously without a condensing agent in an aqueous solution.

Chung *et al.*[11] suggested that the pathway leading to the peptide bond formation involves the intermediate formation of a five-membered cyclic anhydride. However, the mechanism of the formation of peptide was only based on assumption and there was very little experimental data to support it. To disclose the mechanism of the formation of dipeptides by phosphorylation of amino acids with P_{3m}, the structures and the amounts of the reaction products and intermediates, including polyphosphates, peptides, and phosphorylated derivatives, should be determined; but these have been hard to determine accurately, because of the lack of analytical tools for the selective and sensitive characterization of these compounds. During the last decade, new analytical techniques for these compounds have been rapidly developing, *e.g.*, HPLC coupled with a post-column reaction detector,[12-14] a high magnetic field NMR spectroscopy,[15-17] and capillary electrophoresis (CE).[18,19]

In the present study, phosphorylation of several amino acids with P_{3m} was investigated to determine the intermediate formation of a five-membered cyclic anhydride as the key compound in the dipeptide formation. More improved structural diagnosis was made of the reaction products and intermediates using modern analytical techniques.

Results and Discussion

The Reaction of Glycine with P_{3m} Figure 1 shows HPLC profiles for the products in the reaction of glycine (2.5 mol dm^{-3}) with P_{3m} (0.5 mol dm^{-3}) at pH 12 and room temperature. As shown, two peaks due to the reaction products were observed at the retention times of about 11 min (peak A) and 25 min (peak B) after 30 min incubation. The area of peak B decreased gradually and disappeared after 1 d, whereas that of peak A gradually increased with the passage of reaction time. From the results of ^{31}P-NMR as described later, peak A was found to be an unseparated peak for mixture of monophosphate derivatives of glycine or glycylglycine (**1a**, **2a**, and **3a** in Figs. 2 and 3), and peak B was a triphosphate derivative of glycine (**4a**). Other chromatographic peaks were assigned to P_{3m} and inorganic diphosphate (P_2) on the basis of the retention times for the authentic samples.

To establish the structures of the phosphorylated products, ^{31}P-NMR spectra were measured. Figures 2 and 3 show ^{31}P-NMR spectra for the reaction mixture of glycine (2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) incubated at room temperature and pH 12 for 30 min and 4 d, respectively. A proton-decoupling spectrum in Fig. 2 shows several unknown signals, *i.e.*, two doublets (−0.37, −4.5 ppm), a triplet (−19.6 ppm), and three singlets (**2a**: 13.6, **1a**: 9.2, **3a**: 8.9 ppm) in addition to the signals assigned to P_{3m} at −21.4 ppm, P_1 at 5.0 ppm, and P_2 at −4.7 ppm on the basis of the chemical shifts for the authentic samples.

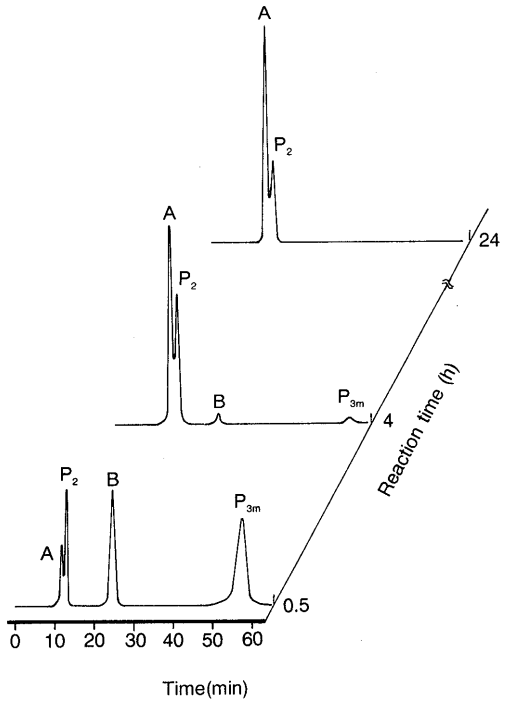


Fig. 1. HPLC Profiles for the Reaction Products of Glycine with P_{3m}
P_{3m}: Gly = 1 : 5 (0.5 mol dm^{-3} : 2.5 mol dm^{-3}), pH 12 and room temperature.

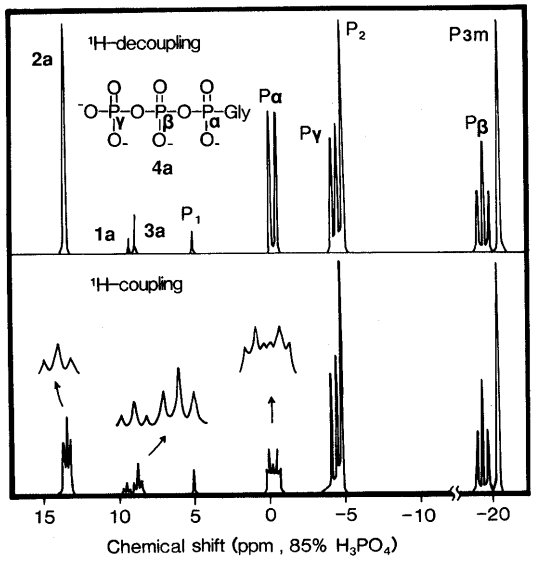


Fig. 2. ^{31}P-NMR Spectra for the Reaction Products of Glycine with P_{3m}
P_{3m}: Gly = 1 : 5 (0.5 mol dm^{-3} : 2.5 mol dm^{-3}), pH 12 and room temperature for 30-min incubation.

Two unknown doublets and an unknown triplet were assigned to the signals for *N*-(carboxymethyl)triphosphoramidate (P_3-(N)Gly: **4a**), $^{-}O\text{-}\overset{O_-}{\overset{\|}{P}}\text{-}O\text{-}\overset{O_-}{\overset{\|}{P}}\text{-}O\text{-}\overset{O_-}{\overset{\|}{P}}\text{-}NHCH_2COO^-$, as shown in Fig. 2 on the basis of the following interpretation of ^{31}P-NMR spectra. The triplet at −19.6 ppm is characteristic of the middle-group phosphorus atom (P_β) on triphosphate species such as ATP.[20,21] Such a triplet around −20 ppm was never observed in the ^{31}P-NMR spectra of mono-, di-, and tetraphosphate species. In addition, the coupling constant (19.3 Hz) of the triplet is equal to that (19.3 Hz) of ATP,[21] the value of which represents the coupling between two phosphorus atoms separated in a P–O–P linkage.[22] The triplet at −19.6 ppm, therefore, is assigned to the middle phosphorus atom (P_β) on **4a**. The unknown doublet at −0.37 ppm is assigned to the signal of P_α atom on **4a**, because the proton coupling spectrum shows that the doublet is split into the double triplet ($^3J_{PH}$ = 6.7 Hz) caused by the coupling between P_α atom and two hydrogen atoms of methylene group on glycine. An unknown doublet at −4.5 ppm, therefore, is assigned to P_γ on **4a**. The amidation of a phosphorus group (P_α) leads to a downfield shift of δ relative to the free phosphorus group (P_γ). The coupling constants between P_α and P_β and between P_β and P_γ were 19.6 and 19.0 Hz, respectively.

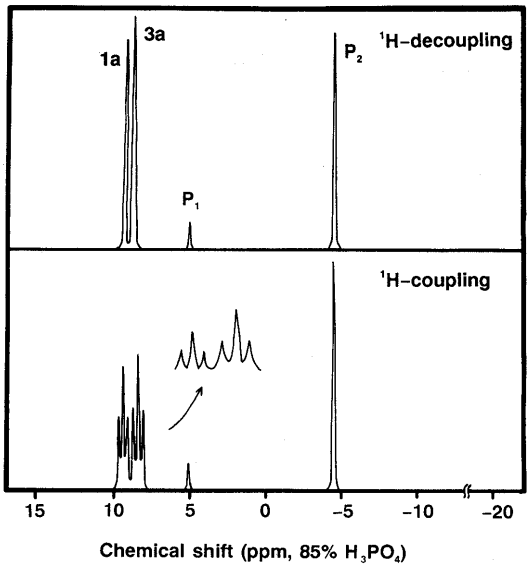


Fig. 3. ^{31}P-NMR Spectra for the Reaction Products of Glycine with P_{3m}
P_{3m}: Gly = 1 : 5 (0.5 mol dm^{-3} : 2.5 mol dm^{-3}), pH 12 and room temperature for 4-d incubation.

Three singlets at 13.6, 9.2, and 8.9 ppm could be assigned to three kinds of monophosphate derivatives (**1a**, **2a**, and **3a**) of glycine or glycylglycine because these signals showed no coupling with other phosphorus atoms. As shown in Fig. 3, the peaks of the products **4a** and **2a** completely disappeared after 4-d incubation, whereas the intensity of the peaks of the other products **1a** and **3a** fairly increased, illustrating that products **4a** and **2a** were formed at an early stage of the reaction and converted into products **1a** and **3a** with reaction time.

Figure 4 shows ^{31}P-NMR spectra for the reaction products of glycylglycine (2.5 mol dm^{-3}) with P_{3m} (0.5 mol dm^{-3}) incubated at room temperature and pH 12 for 5 d. Four peaks corresponding to the reaction products were observed at −1.2 ppm (doublet), −4.5 ppm (doublet), −19.8 ppm (triplet), and 8.9 ppm (singlet) in the proton-decoupling spectrum. The products were identified as *N*-(*N*-phosphonoglycyl)glycine (P_1-(N)GlyGly, δ = 8.9) and *N*-(*N*-triphosphonoglycyl)glycine (P_3-(N)GlyGly, δ = −1.2, −4.5, −19.8). The triplet at −19.8 ppm was assigned to the middle phosphorus atom (P_β on P_3-(N)GlyGly) similar to the case of the phosphorylation of glycine as described above. The doublets at −1.2 and −4.5 ppm were assigned

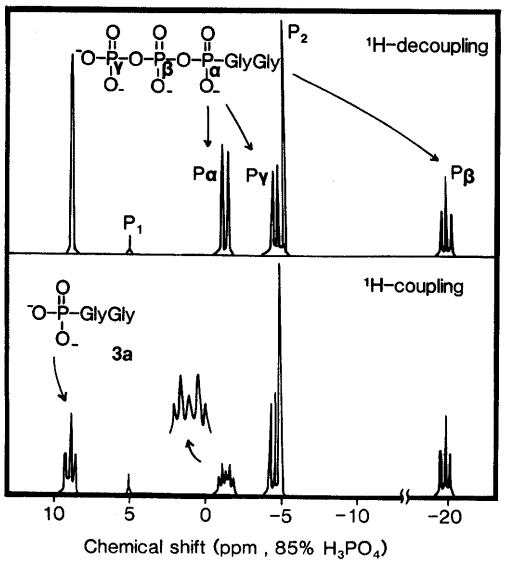


Fig. 4. ^{31}P-NMR Spectra for the Reaction Products of Glycylglycine with P_{3m}

P_{3m} : GlyGly = 1 : 5 (0.5 mol dm^{-3} : 2.5 mol dm^{-3}), pH 12 and room temperature for 5-d incubation.

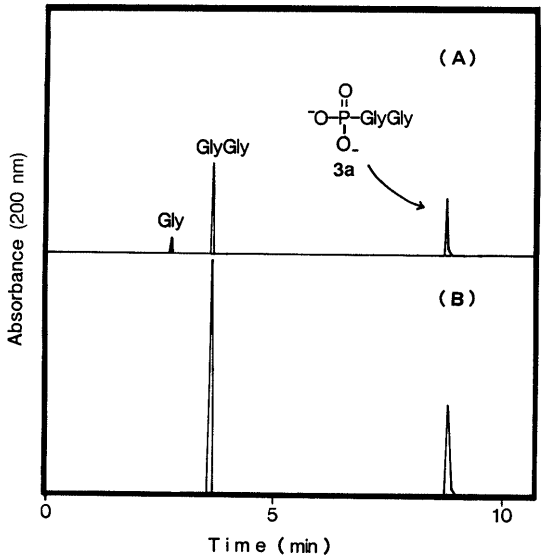


Fig. 5. CE Separation of the Reaction Products of Glycine (A) or Glycylglycine (B) with P_{3m} for 7-d Incubation

to the signals for P_α and P_γ atoms on P_3-(N)GlyGly, respectively. Product **3a** in Fig. 3 can be identified as P_1-(N)GlyGly, because the value of chemical shift of P_1-(N)GlyGly ($\delta = 8.9$) in Fig. 4 is exactly the same as that of **3a** ($\delta = 8.9$).

The singlet at 9.2 ppm (**1a**) split into the triplet ($^3J_{PH} = 6.3$ Hz) in the proton-coupling spectrum as shown in Fig. 3. The coupling constant (6.3 Hz) is approximately equal to that ($^3J_{PH} = 6.7$ Hz) of **4a**. Thus, product **1a** would be *N*-(carboxymethyl)phosphoramidate (P_1-(N)-Gly), $^-O\text{-}\overset{\overset{O}{\|}}{P}\text{-}NHCH_2COO^-$, or 1-aminoacetyl phosphate, $^-O\text{-}\overset{\overset{O}{\|}}{P}\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_2\text{-}NH_2$.

^{31}P-NMR data of acetylphosphate, $^-O\text{-}\overset{\overset{O}{\|}}{P}\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3$, which is similar to 1-aminoacetyl phosphate in structure showed a singlet peak at $\delta = -2.0$.[23] The δ-value of 1-aminoacetyl phosphate, which would be similar to the δ-value of acetylphosphate, is quite different from that (9.2 ppm) of **1a**. On the other hand, product **1a** gave a triplet in a proton coupling spectrum, whereas there was no coupling between a phosphorus and hydrogen atoms on acetylphosphate. Therefore, product **1a** is assigned to P_1-(N)Gly and the formation of 1-aminoacetyl phosphate was excluded in the reaction of P_{3m} with glycine.

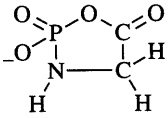
The singlet (**2a**) at 13.6 ppm in Fig. 2 was assigned to a five-membered cyclic phosphate,  , on the basis of experimental and theoretical work[24,25] by Gorenstein on the ^{31}P-NMR chemical shifts on O–P–O bond angles in monophosphate species. His conclusion is that the δ-values of five-membered cyclic phosphates shift downfield relative to the corresponding monophosphates, the δ-values of which are around 0—3 ppm, by 15—20 ppm, and such a downfield shift is caused by a decrease in the O–P–O bond angle of five-membered cyclic phosphates. Therefore, five-membered cyclic phosphate (**2a**) was assigned to be 5-oxo-1,3,2-oxazaphospholidine-2-olate 2-oxide. Although we attempted to isolate the product **2a**, we were unsuccessful because of its instability.

Figure 5 shows CE profiles for the reaction product of glycine (Fig. 5A, 2.5 mol dm^{-3}) or glycylglycine (Fig. 5B, 2.5 mol dm^{-3}) and P_{3m} (0.5 mol dm^{-3}) incubated at room temperature and pH 12 for 7 d. In Fig. 5A three peaks were observed. By comparing with the migration times of the authentic samples, the two peaks at 2.9 and 3.5 min were assigned to Gly and GlyGly, respectively. Figrue 5B gave two peaks of GlyGly and the reaction product (**3a**). From these results, the formation of product **3a** was confirmed in the reaction of glycine with P_{3m}. Also, product **3a** was easily hydrolyzed to GlyGly and P_1.

Figure 6 shows the changes in the amounts of the products formed by the reaction of P_{3m} (0.5 mol dm^{-3}) with glycine (2.5 mol dm^{-3}) at pH 12 and room temperature. The quantitative analyses of the products (**1a**, **2a**, **3a**, and **4a**) were performed by HPLC, and that of GlyGly by CE. The amount of **4a** drastically increased, reaching about 22% after 30 min, and after that decreased with time. The total amounts of monophosphate derivatives (**1a**, **2a**, and **3a**) of glycine gradually increased with the reaction time and reached about 71% after 1 d. Starting material, P_{3m}, decreased with time, and disappeared after 1 d. Glycylglycine was formed at an early stage of the reaction, and its yield was 9.2% after 1 d. The reaction of glycine with P_{3m} was further carried out by varying reaction conditions (pH and temperature). The optimum conditions for the maximum formation of **1a**, **2a**, **3a**, and **4a** were found to be high mixing ratio of glycine to P_{3m} (5 : 1), high pH (12), and room temperature (Table I).

Table II lists the maximum yields of the phosphorylated products and dipeptides in the reactions of amino acids

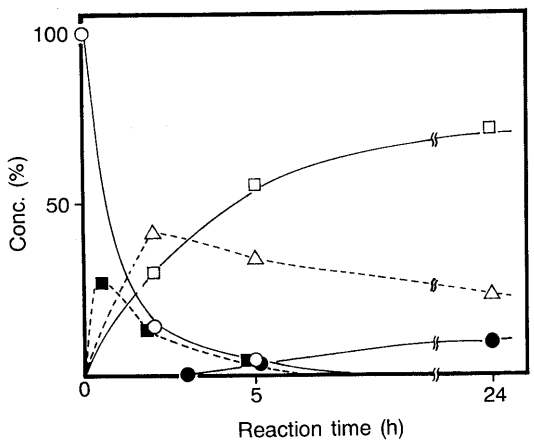


Fig. 6. Change of the Amounts of Reaction Products of P_{3m} (0.5 mol dm^{-3}) with Glycine (2.5 mol dm^{-3}) at pH 12 and Room Temperature

—○—: P_{3m}, —□—: monophosphate derivatives (**1a**, **2a** **3a**), --△--: P_2, —●—: GlyGly, --■--: triphosphate derivative (**4a**).

Table I. Yields of Phosphorylated Products in the Reaction of Glycine with P_{3m}

Reaction conditions			Yield (%) as P	
Mixing ratio/mol dm^{-3} P_{3m} : Gly	pH	Temp. (°C)	**1a**, **2a**, **3a**	**4a**
0.5 M : 2.5 M	12	Room temp.	71	22
0.5 M : 2.5 M	12	50	31	0.8
0.5 M : 2.5 M	12	70	29	0.6
0.5 M : 2.5 M	10	Room temp.	43	11
0.5 M : 2.5 M	7	Room temp.	0	0

Table II. Yields of Phosphorylated Products and Dipeptides

Reaction condition	Yield (%) as P		Dipeptide
Mixing ratio/mol dm^{-3}	P_1-der.	P_3-der.	
P_{3m} : Gly = 0.5 : 2.5	71 (**1a**, **2a**, **3a**)	22 (**4a**)	15.8 (**5a**)
P_{3m} : Ala = 0.5 : 1.5	50 (**1b**, **2b**, **3b**)	2 (**4b**)	2.0 (**5b**)
P_{3m} : Val = 0.5 : 0.5	22 (**1c**)	0	0
P_{3m} : Ser = 0.5 : 2.5	58 (**1d**)	0	0

Table III. ^{31}P-NMR Data for Monophosphate Derivatives

Product	δ(P)	$^3J_{PH}$/Hz
1a	9.2	6.3
2a	13.6	8.0
3a	8.9	9.6
1b	9.4	9.7
2b	12.2	9.8
3b	8.7	11.5
1c	9.0	10.5
1d	8.6	8.8

Table IV. ^{31}P-NMR Data for Triphosphate Derivatives

Product	$\delta(P_\alpha)$	$\delta(P_\beta)$	$\delta(P_\gamma)$	$^2J_{P_\alpha P_\beta}$/Hz	$^2J_{P_\beta P_\gamma}$/Hz	$^3J_{P_\alpha H}$/Hz
4a	−0.37	−19.6	−4.5	19.6	19.0	6.7
4b	−0.38	−18.8	−3.5	19.7	19.2	9.0

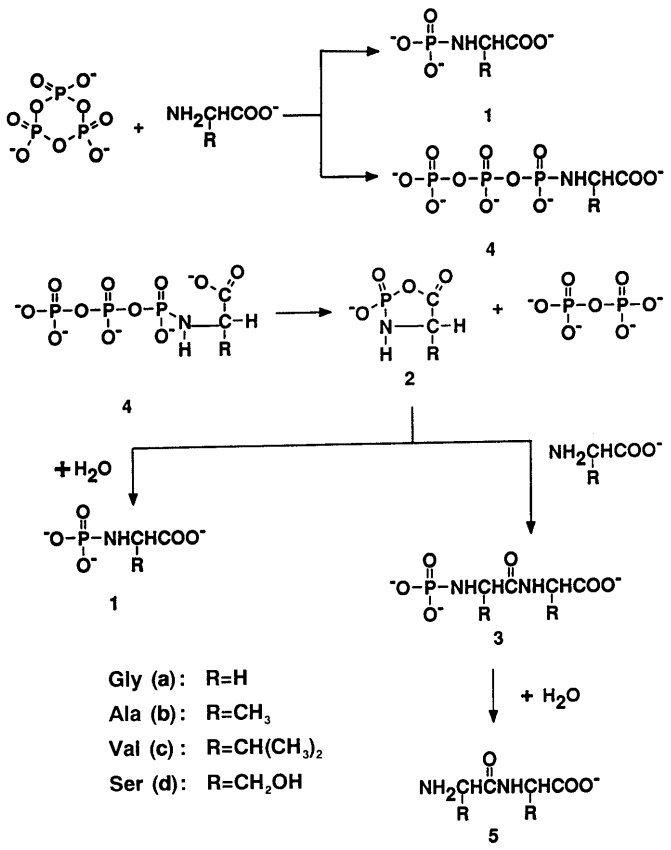


Fig. 7. Mechanism of the Reaction in Phosphorylation of Amino Acids with P_{3m}

(glycine, alanine, valine, and serine) with P_{3m}. The yield of GlyGly (**5a**) was 15.8% after 18 d of reaction.

The Reaction of Ala with P_{3m} Tables III and IV list data of ^{31}P-NMR for the phosphorylated products (monophosphate and triphosphate derivatives) in the reactions of amino acids with P_{3m}. As shown in these tables and Table II, the kinds and structures of the reaction products of Ala with P_{3m} corresponded to those of Gly with P_{3m}. From the results of ^{31}P-NMR, the structures of phosphorylated products were determined to be *N*-(1-carboxyethyl)phosphoramidate (P_1-(N)Ala: **1b**), 4-methyl-5-oxo-1,3,2-oxazaphospholidin-2-olate 2-oxide (P_1-(N,O)Ala: **2b**) of five-membered cyclic anhydride, *N*-(*N*-phosphonoalanyl)alanine (P_1-(N)AlaAla: **3b**), and *N*-(1-carboxyethyl)triphosphoramidate (P_3-(N)Ala: **4b**). From Table II, the yields of monophosphate derivatives (**1b**, **2b**, and **3b**) and triphosphate derivative (**4b**) of Ala, and dipeptide (AlaAla: **5b**) were 50, 2.0, and 2.0%, respectively. The amount of AlaAla (2.0%) was smaller than that of GlyGly (15.8%). The yields of dipeptide might be relative to the amounts and stability of five membered cyclic phosphates formed in the reaction of Gly or Ala with P_{3m}.

The Reactions of Val, and Ser with P_{3m} The reactions of Val, and Ser with P_{3m} gave no dipeptides. The products of these reactions were only *N*-(1-carboxy-2-methylpropyl)-phosphoramidate (P_1-(N)Val: **1c**),[26] and *N*-(1-carboxy-2-hydroxyethyl)phosphoramidate (P_1-(N)Ser: **1d**), respectively. On the other hand, a by-product in the reactions of Val and Ser with P_{3m} was inorganic diphosphate (P_2), suggesting that triphosphate derivatives (**4c** and **4d**) of Val

and Ser were very unstable and immediately hydrolyzed to monophosphate derivatives (**1c** and **1d**) and P_2. The ^{31}P-NMR parameters of products **1c** and **1d** are shown in Table III.

Glycine and alanine reacted with P_{3m} to yield their dipeptides, whereas dipeptide was not obtained in the reactions of Val and Ser with P_{3m} at all (Table II).

Mechanism of the Reaction of Amino Acid with P_{3m} The phosphorylation of amino acids with P_{3m} and the formation of dipeptides were examined in this study using new analytical techniques. The reaction products and intermediates (five-membered cyclic phosphates) were detected by HPLC, ^{31}P-NMR, and CE. The mechanism of the formation of dipeptide was proposed as shown in Fig. 7.

Amino group of amino acid nucleophilically attacks a phosphorus atom on P_{3m} to give the product **4**. An intramolecular attack of carboxyl group on a phosphorus atom of **4** yields five-membered cyclic phosphate (product **2**). The reaction of **2** with excess amino acids gives the product **3**, which is easily hydrolyzed to dipeptide (**5**). Hydrolysis of **2** occurs simultaneously to form the product **1**. In the case of formation of dipeptide, five-membered cyclic phosphate (**2**) should be stability and a strong electrophilic compound. Although a number of synthetic routes of dipeptide formation have been proposed in the literature,[3,5,11] none of these elucidate the existence of compounds **4** and **2** in the reactions of amino acids with P_{3m}. Here, we were able to show that amino acids (glycine and alanine) react with P_{3m} to form the intermediates **2** and **4**, and also that the intermediate **2** is the key compound for the production of dipeptide. Our experimental results may suggest that the simple formation of dipeptide in an aqueous solution correlates to the origin of life.

Experimental

Chemicals Sodium *cyclo*-triphosphate hexahydrate (P_{3m}), $Na_3P_3O_9 \cdot 6H_2O$, was purchased from Rasa Industries, Ltd. (Osaka, Japan) and recrystallized from an aqueous solution. The purity of P_{3m} thus obtained was checked by HPLC to be 99% (as P). Guaranteed grade glycine, glycylglycine, L-alanine, L-alanyl-L-alanine, L-valine, and L-serine were purchased from Wako Chemical Industries, Ltd. (Osaka, Japan).

Reaction of Amino Acids with P_{3m} in an Aqueous Solution The initial concentration of P_{3m} was kept at a constant value of 0.5 mol dm^{-3} and those of glycine and serine at 2.5 mol dm^{-3}. Alanine reacted with P_{3m} at the fixed mixing ratio of 3 : 1 (1.5 mol dm^{-3}: 0.5 mol dm^{-3}). The reaction of valine with P_{3m} was carried out at the fixed mixing ratio of 1 : 1 (0.5 mol dm^{-3}: 0.5 mol dm^{-3}). Since the pH of the mixed solution was gradually decreasing with the progress of the reaction, it should be adjusted to the prescribed pH (12, 10, 7) by adding 6 mol dm^{-3} sodium hydroxide solution. The mixed solutions were allowed to react at room temperature (20—25 °C) or at specified temperatures (50, 70 °C) controlled by a thermostated bath within ±2 °C.

HPLC Measurement HPLC analysis was carried out with a JASCO Tri-rotar VI HPLC system (Tokyo, Japan) coupled with a JASCO FIU-300 flow injection system to detect phosphate as a post-column reaction detector. A column (250 × 4.6 mm i.d.) was packed with a polystyrene-based anion-exchanger (TSK gel, SAX, 10 μm, Tosoh, Tokyo, Japan) and the column temperature was maintained at 40 °C. An aliquot (0.1 ml) of the reaction mixture was injected. Flow rate was 1.0 ml min^{-1}. The convex gradient elution technique using 0.2 and 0.45 mol dm^{-3} potassium chloride solutions was used. Determination of phosphates (ortho-, pyro-, and triphosphates) and phosphate groups on the products were carried out with spectrophotometry of phosphorus-molybdenum heteropoly blue complex. The absorbance of an effluent was continuously monitored at 830 nm.

^{31}P-NMR Measurement Pulse FT ^{31}P-NMR spectra were recorded at room temperature using a Varian XL-VX 200 (81 MHz) spectrometer. Orthophosphoric acid (85%) was used as an external standard.

CE Capillary electrophoretic separations were made by a Waters Quanta-4000 CE system and an Applied Biosystems Inc. (ABI) Model 270 A CE system. Polyimide coated fused silica capillary (375 μm o.d., 50 μm i.d., Polymicro Technologies, Phoenix, Az, U.S.A.) of 50 cm effective length and 70 cm total length was used without its inner surface pretreatment. Buffer was 0.2 mol dm^{-3} sodium borare (pH 8) or 0.1 mol dm^{-3} phosphate (pH 2.5). Sample solution was introduced using a vacuum injection system (1 s). Applied voltage was 30 kV (429 V/cm, 28 μA) and the capillary temperature was controlled at 30 °C (ABI) and room temperature (Quanta-4000). The reaction products and dipeptide were detected at 185 nm (Quanta-4000) and 200 nm (ABI).

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