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Divalent Metal Ion-Mediated Phosphodiester Bond Formation from Adenosine-5′-phosphorimidazolide with Glycolic Acid or Lactic Acid in Aqueous Solution. A Nonenzymatic Model Reaction for Nucleotidyl Transfer

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Some divalent metal ions mediated in phosphodiester bond formation from adenosione-5′-phosphorimidazolide (ImpA) and glycolic acid or lactic acid giving glycolyl adenylate or lactyl adenylate. The effect of divalent metal ions on this reaction was in the order: $Pb^{2+}>Ni^{2+}>Co^{2+}>Zn^{2+}>Cu^{2+}>Mn^{2+}>Ca^{2+}>Mg^{2+}>$ none$>Hg^{2+}$. The metal ions promoted the reaction orienting the substrates and increasing the nucleophilicity of the hydroxy group of α-hydroxy acid by coordination. On the other hand, very little or no phosphodiester bond formation took place from ImpA and 3-hydroxypropionic acid or 4-hydroxybutyric acid in the presence of metal ions.

Nucleotidyl and phosphate transfer reactions play key roles in biological systems such as bioenergy metabolism and biosynthesis. The enzymes which catalyze these reactions require divalent metal ions such as Mg^{2+}, Mn^{2+}, or Zn^{2+} for their activity.1) The role of divalent metal ions in enzymatic activity has been of considerable interest. It has been postulated that metal ions work as; (i) a kind of template for the precise alignment of substrates into enzymic catalytic site, (ii) a factor in promoting the nucleophilicity of the hydroxyl group of the substrate by coordination, and (iii) a factor in the charge neutralization of the phosphate group.1) Several nonenzymatic model reactions have been performed by using divalent metal ions to study the catalytic role of divalent metal ions in enzymatic phosphoryl transfer reactions. Cooperman and his research group reported that Zn^{2+} ion catalyzes the phosphoryl transfer from phosphoryl-imidazole (PIm) to pyridine-2-carbaldehyde oximate (PCA) anion via the formation of a PCA–Zn^{2+}–PIm ternary complex.2) Further Sigman and his group found that Zn^{2+} ion catalyzes the phosphorylation of 2-hydroxymethyl-1,10-phenanthroline by ATP through the formation of a reactive ternary complex which favors the phosphate transfer.3) On the other hand, Lowenstein and his group stated that the transfer of the phosphoryl group from ATP to acetate and inorganic phosphate is catalyzed by divalent metal ions.4) Recently nonenzymatic phosphorylation of peptides in the hydroxyl group of tyrosine and serine by ATP has also been reported.5) The phosphoryl transfer reaction is catalyzed by Mn^{2+} ion but not by Mg^{2+} ion in neutral aqueous solution. In these reactions, divalent metal ions increase the rate of phosphate transfer to a greater extent than that of the hydrolysis of PIm or ATP.

Very little has been reported about metal ion-catalyzed nucleotidyl transfer reactions. Previously we showed that metal ions such as Zn^{2+}, Pb^{2+}, and UO_2^{2+} catalyze the condensation of ImpA in aqueous solution to form oligoadenylic acids.6,7) We postulated that metal ions served as a template orienting ImpA by coordination and enhanced the nucleophilicity of the 2′- or 3′-hydroxyl group of ImpA, thereby facilitating the adenylyl group transfer to form an internucleotide linkage. In this paper I further examined the effects of the metal ions on adenylyl group transfer from ImpA to hydroxyl group of glycolic acid or lactic acid in neutral aqueous solution.

Experimental

Materials: Adenosine-5′-phosphorimidazolide (ImpA) was prepared by modifying the published procedure.8) *N*-Ethylmorpholine, from Tokyo Kasei, was distilled before use. Other reagents were obtained commercially and used without further purification.

Standard Procedure for the Reaction of ImpA with Hydroxy Acid: A reaction mixture containing ImpA (25 mM; 1 M=1 mol dm^{-3}), glycolic acid (50 mM), and metal chloride or metal nitrate (25 mM) in a 0.2 M *N*-ethylmorpholine buffer (pH 7.0 or 8.0) (0.1 ml) was kept at 25 °C. When the reaction was carried out at pH 5.0, 6.0, and 9.0, no buffer was used and the pH value of the reaction mixture was adjusted with 0.1 M NaOH or 0.1 M HCl solution through a microcapillary tube. The solutions were withdrawn after 1—10 d and treated with 20 μl of 0.25 M EDTA or Versenol solution to break down the nucleotide-metal complex. The reaction mixtures were then subjected to analysis by high performance liquid chromatography (HPLC).

The reactions of ImpA with lactic acid, 3-hydroxypropionic acid or 4-hydroxybutyric acid were carried out in the same way as described above, and the samples were analyzed by HPLC.

The reaction of ImpA (25 mM) with methanol (50 mM) was performed in the presence of zinc chloride or lead(II) nitrate (25 mM) in 0.2 M *N*-ethylmorpholine buffer (pH 7.0) (0.1 ml) at room temperature. This reaction was also done in the presence of sodium acetate (50 mM) to examine the effect of carboxyl group on the phosphodiester bond formation.

High Performance Liquid Chromatography: HPLC was performed using a Hitachi 638 equipped with a RPC-5 column9 (4 mm×25 cm). The elution was carried out with a linear gradient of $NaClO_4$ solution (0—2 mM) buffered with 2 mM Tris–acetate (pH 7.5) and 0.1 mM EDTA in 20 min at a flow rate of 1.0 ml min^{-1}. The eluate was monitored by UV absorption at 260 nm. Yields were calculated from peak integrals of the reaction products on the HPLC chromatograms. No correction for hypochromicity of each compound was done in the calculation.

Characterization of the Products: The products were identified by comparing the HPLC chromatogram with that of the authentic samples. Glycolyl adenlate was isolated from the reaction mixture of ImpA (25 mM), glycolic acid (50 mM), and lead(II) nitrate in 0.2 M *N*-ethylmorpholine buffer (pH 7.0) (5 ml) after 2 d at 23 °C. The mixture was treated with 0.25 M EDTA solution and subjected to QAE-Sephadex A-25 column chromatography with a linear gradient elution consisting of triethylammonium hydrogencarbonate buffer (pH 7.5) [0.0 M (500 ml)—0.5 M (500 ml)]. The appropriate UV absorbing fractions were collected and lyophilized. The isolated yield was 77% based on the starting ImpA. The structure of GA-pA was confirmed by NMR and IR. ^1H NMR in D_2O; δ=8.46 (1H, 8-H), 8.20 (1H, 2-H), 6.15 J=5.4 Hz (1H, 1′-H), 4.33 (2H, 5′-H), 4.21 (2H, HOOC-CH_2-). IR (KBr); 1070, 1403, 1600. R_f, 0.45 in TLC with 2-propanol–concd NH_3 aq–H_2O (7:1:2) on a Merck cellulose F TLC plate.

Isolation and identification of lactyl adenylate (LA-pA) were carried out by the same procedure employed for those of glycolyl adenylate. ^1H NMR in D_2O; δ=8.50 (1H, 8-H), 8.18 (1H, 2-H) 6.10 J=6.0 Hz (1H, 1′-H), 1.55 J=7.0 Hz (3H, -CH_3).

Authentic 3-hydroxypropionyl adenylate (HA-pA) was prepared by modification of the method described by Chang.[10] Condensation of pA with benzyl 3-hydroxypropionate in dry pyridine using dicyclohexylcarbodiimide as a condensing agent yielded the protected HP-pA, which was subjected to hydrogenolysis on Pd-carbon under the atmospheric pressure of hydrogen gave HA-pA. R_f: 0.59 in TLC with 2-propanol–concd NH_3 aq–H_2O (7:1:2) on a Merck cellulose F TLC plate.

Authentic methyl adenylate was prepared from pA and methanol using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide as a condensing agent.[8] R_f: 0.68 in TLC with 2-propanol–concd NH_3 aq–H_2O (7:1:2) on a Merck cellulose F TLC plate.

Results and Discussion

The reaction of ImpA with glycolic acid was performed in aqueous solution in the presence of divalent metal ions. The reaction mixture was homogeneous with Ca^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, or Mn^{2+} ions, while the use of Pb^{2+}, Zn^{2+}, Hg^{2+}, or Cu^{2+} ions induced precipitation. GA-pA and pA were formed along with small amounts of diadenosine pyrophosphate (AppA) and oligoadenylates ($(pA)_n$). ImpA disappeared as the reaction progressed. Typical HPLC profiles of the reaction mixture, where Pb^{2+} and Zn^{2+} ions were used, are shown in Fig. 1. The HPLC profile of the control experiment, in which no divalent metal ion was present, is also shown in Fig. 1. Table 1 lists the yield data of the reaction of ImpA

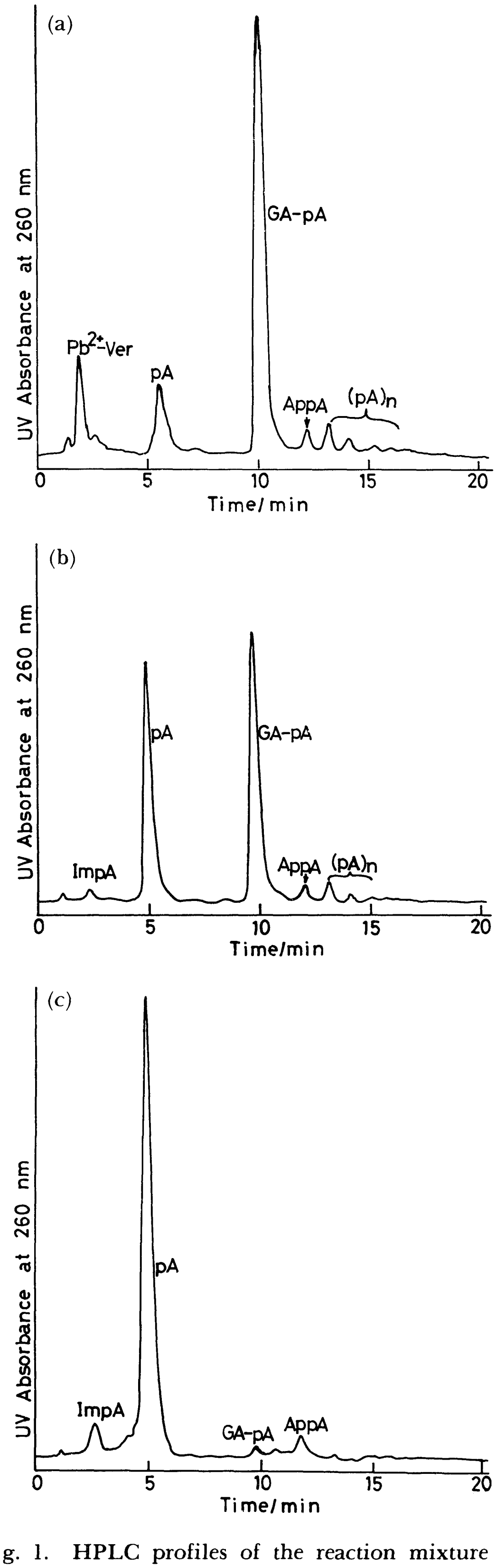

Fig. 1. HPLC profiles of the reaction mixture of ImpA and glycolic acid. (a) The lead(II) ion-mediated GA-pA formation from ImpA and glycolic acid at room temperature for 8 h. (b) The zinc ion-mediated GA-pA formation from ImpA with glycolic acid at room temperature for 7 d. (c) The reaction of ImpA with glycolic acid in the absence of divalent metal ion at room temperature for 7 d.

with glycolic acid. Metal ions such as Pb^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} enhanced the GA-pA formation. The ratio of the yield of GA-pA to that of pA roughly expresses the selectivity of adenylyl group transfer to the hydrolysis of ImpA. Pb^{2+} demonstrated the highest activity and increased the ratio by 150—300 fold compared to the control reaction in which divalent metal ion was omitted. The effect of the metal ions on the reaction was in the following order; $Pb^{2+}>Ni^{2+}>Co^{2+}>Zn^{2+}>Cu^{2+}>Mn^{2+}>Ca^{2+}>Mg^{2+}>$ none$>Hg^{2+}$. The order is the same as that of metal ion-catalyzed oligoadenylate synthesis from ImpA,[6] except for the Zn^{2+}, Mn^{2+}, and Cu^{2+} ions. The similarity of metal ion dependency suggests that the metal ions have analogous roles in both of the reactions. The effect of the pH of the reaction medium on the GA-pA formation is shown in Fig. 2. The efficiency of the phosphodiester bond formation was optimum at pH 7.0 with Pb^{2+} ions, and at pH 8.0 with Zn^{2+} ions.

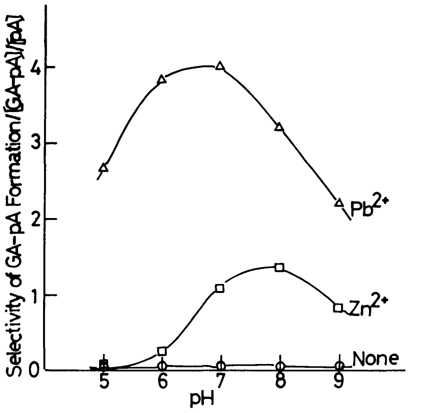

Fig. 2. Effect of pH on GA-pA formation. Selectivity of GA-pA formation, the ratio of yield of GA-pA to that of pA, is shown in the ordinate. A reaction in the presence of $Pb(NO_3)_2$ or $ZnCl_2$ was carried out at 28 °C for 8 h and 1 d for the reaction below 8.0 and 9.0, respectively. Reactions without metal ions were carried out at 23 °C for 1 d and 10 d, for the raction below 7.0 and 9.0, respectively.

Similarly, metal ions such as Pb^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} promoted the phosphodiester bond formation from ImpA and lactic acid giving lactyl adenylate (LA-pA). Table 2 shows the yield data of LA-pA formation. The yields of LA-pA were lower than that of GA-pA, due to the steric effect of the methyl group on lactic acid.

Very little or no phosphodiester bond formation took place from ImpA with 3-hydroxypropionic acid or 4-hydroxybutyric acid in the presence of metal ions as shown in Table 3. The divalent metal ion also did

Table 1. Yields of Products from the Reaction of ImpA with Glycolic Acid

Time	Metal salt	Yield/%					Selectivity [GA-pA]/[pA]
		GA-pA	pA	AppA	$(pA)_n$	ImpA	
8 h	$Pb(NO_3)_2$	74.5	18.6	2.3	3.1	1.5	4.01
8 h	$ZnCl_2$	31.2	29.8	2.4	4.0	32.6	1.05
7 d	$ZnCl_2$	45.6	43.1	3.0	6.8	1.5	1.06
7 d	$NiCl_2$	52.7	26.6	1.9	1.3	17.6	1.98
7 d	$CoCl_2$	45.7	39.4	5.3	4.2	5.3	1.16
7 d	$CuCl_2$	22.2	71.5	2.3	2.8	1.2	0.31
7 d	$MnCl_2$	8.5	52.6	23.0	8.8	7.1	0.16
7 d	$CaCl_2$	1.6	70.9	13.7	2.9	10.9	0.02
7 d	$MgCl_2$	1.3	77.0	12.0	1.4	8.3	0.02
7 d	None	1.2	88.5	3.5	0.7	6.1	0.01

Reactions were run at 23 °C and pH 7.5. 25 mM ImpA, 50 mM glycolic acid, and 25 mM metal chloride were used.

Table 2. Yields of Products from the Reaction of ImpA with Lactic Acid

Time	Metal chloride	Yield/%					Selectivity [LA-pA]/[pA]
		LA-pA	pA	AppA	$(pA)_n$	ImpA	
1 d	$Pb(NO_3)_2$	37.1	40.8	2.1	17.1	2.9	0.1
1 d	$ZnCl_2$	7.4	59.9	3.9	10.9	17.9	0.12
1 d	$CuCl_2$	8.7	77.2	5.3	2.8	6.0	0.11
7 d	$NiCl_2$	7.9	53.1	3.3	2.8	32.8	0.15
7 d	$CoCl_2$	8.2	60.1	6.7	14.1	10.9	0.14
7 d	MgCl	0.4	77.7	12.0	1.7	7.9	0.005
7 d	None	0.4	86.5	3.2	1.6	8.3	0.005

Reactions were run at 23 °C and pH 7.5. 25 mM ImpA, 50 mM lactic acid, and 25 mM metal chloride were used.

Table 3. Yields of Products from the Reaction of ImpA with 3-Hydroxypropionic Acid or 4-Hydroxybutyric Acid

Time	Metal chloride	Yield/%				
		HA-pA	pA	AppA	$(pA)_n$	mpA
3-Hydroxypropionic Acid						
1 d	$Pb(NO_3)_2$	3.6	42.6	2.5	45.2	6.1
7 d	$ZnCl_2$	1.4	73.2	3.1	19.2	3.1
7 d	$MgCl_2$	tr	73.1	18.3	0.7	7.9
7 d	None	tr	87.5	3.6	1.1	7.8
4-Hydroxybutyric Acid						
1 d	$Pb(NO_3)_2$	3.5	50.3	2.8	41.9	1.5
7 d	$ZnCl_2$	0.5	72.7	4.3	20.7	2.2
7 d	None	tr	88.6	4.0	2.0	5.2

Reactions were run at 23 °C and pH 7.0. 25 mM ImpA, 50 mM hydroxyacid, and 25 mM metal chloride were used.

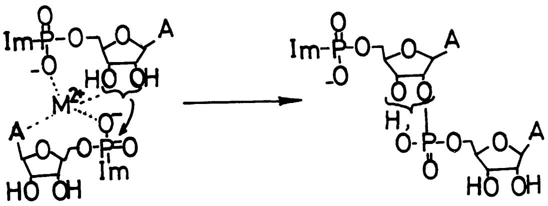

Chart 1. Proposed scheme for metal ion-mediated internucleotide linkage formation.

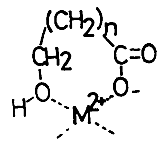

Chart 3.

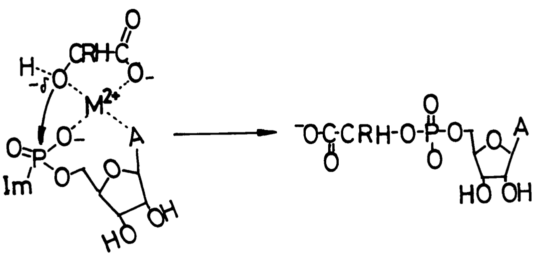

Chart 2. Proposed scheme for metal ion-mediated GA-pA formation.

not mediate methyl adenylate formation from ImpA and methanol in neutral aqueous solution.

These results suggest that GA-pA is formed via a ternary complex composed of glycolic acid, ImpA and metal ions. Metal ion orients the ImpA and glycolic acid, thereby facilitating the adenylyl group transfer. Glycolic acid coordinates with the metal ions such as Pb^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} with its carboxyl and hydroxyl groups forming five-membered chelate ring. The chelation with carboxyl group can promote the coordination and deprotonation of hydroxyl group of α-hydroxy acid. The metal ions, therefore, increase the nucleophilicity of the hydroxyl group of glycolic acid. The hydroxyl group activation involved in the five membered chelate ring formation is also possible in the case of lactic acid.

On the other hand, in the case of 3-hydroxypropionic acid or 4-hydroxybutyric acid, metal ions could not make a proper orientation of the reaction site to activate the hydroxyl group favorable for phosphodiester bond formation, since six or seven-membered chelate rings as in chart 3 are unlikely. The chelate effect can account to the stability constants of metal complexes. It has been established that five-membered chelate rings are more stable than comparable six-membered rings, providing no resonance effects are involved.[11] There are few complexes known with seven-membered chelate rings. The weak coordinating ability of hydroxyl group could make impossible the six-membered chelate ring. The importance of the hydroxyl activation by the metal-chelate for the phosphodiester bond formation was also indicated by the fact that no methyl adenylate was obtained from ImpA and methanol in neutral aqueous solution in the presence of lead(II) nitrate or zinc chloride.

A number of enzymes catalyzing phosphoryl or nucleotidyl group transfer require Zn^{2+} for their activity.[1] One possible role for Zn^{2+} in the catalytic reaction is in coordinating and promoting the nucleophilicity of the substrate and securing the proper orientation of enzyme and the substrate. The Zn^{2+} ion in the enzymatic reactions plays a comparable mechanistic role to the metal ions in the adenylyl group transfer from ImpA to hydroxyl group of GA and from the ImpA to 2′- or 3′-hydroxyl group of another ImpA.[6] The Zn^{2+} ion can be removed from some of the zinc enzymes which catalyze the transfer of phosphates and nucleotides. Replacement of Zn^{2+} by divalent metal ions such as Co^{2+} is possible, although the catalytic activities will be more or less lost.[12)]

Pb^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+} were highly active in the non-enzymatic adenylyl transfer reactions. Among the metal ions we studied, Pb^{2+} showed the highest activity. This ion presumably increases the nucleophilicity of the hydroxyl group of the substrate more efficiently than any other metal ions.

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References

1) J. R. Knowels, *Ann. Rev. Biochem.*, **49**, 877 (1980); M. F. Dunn, *Structure and Bonding*, **23**, 61 (1975); A. S. Mildvan, *Adv. Enzymol.*, **49**, 103 (1979).

2) G. J. Lloyd and B. S. Cooperman, *J. Am. Chem. Soc.*, **93**, 4883 (1971).

3) D. S. Sigman and C. T. Jorgensen, *J. Am. Chem. Soc.*, **94**, 1724 (1972).

4) M. Tetas and J. Lowenstein, *Biochemistry*, **2**, 350 (1963).

5) G. Schieven and G. S. Martin, *J. Biol. Chem.*, **263**, 15590 (1988).

6) H. Sawai, *J. Am. Chem. Soc.*, **98**, 7037 (1976).

7) H. Sawai, K. Kuroda, and T. Hojo, *Bull. Chem. Soc. Jpn.*, **62**, 2018 (1989).

8) R. Lohrmann and L. E. Orgel, *Tetrahedron*, **34**, 853 (1978).

9) H. Sawai, *J. Chromatogr.*, **481**, 201 (1989).

10) P. K. Chang, "Nucleic Acid Chemistry," Part 2, ed by L. B. Townsend and R. S. Timpson, John Wiley & Sons, New York (1978), p. 847.

11) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," John Wiley & Sons, New York (1962), p. 545.

12) B. L. Vallee and A. Galdes, *Adv. Enzymol. Relat. Area Mol. Biol.*, **56**, 284 (1984).