Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 396-402

DIFFERENT ROLES OF THE DIPHOSPHATE MOIETIES OF ALLYLIC AND HOMOALLYLIC DIPHOSPHATES IN PRENYLTRANSFERASE REACTION

Takeshi Gotoh, Tanetoshi Koyama, and Kyozo Ogura

Chemical Research Institute of Non-Aqueous Solutions,
Tohoku University, Katahira 2-1-1, Sendai 980, Japan

Received August 22, 1988

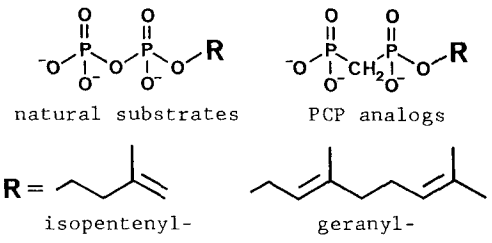
SUMMARY: In contrast to the reactivity of geranyl methylene-diphosphonate in the reaction catalyzed by farnesyl diphosphate synthase, that of isopentenyl methylenediphosphonate showed an optimum at a more acidic pH than that of isopentenyl diphosphate, and it was inhibited by magnesium ions under certain conditions. These facts suggest that isopentenyl diphosphate is engaged in the enzyme reaction in the form of metal-free substrate contrary to the allylic substrate, which reacts in the form of metal-complexed substrate. Thus the diphosphate moieties of allylic and homoallylic substrates have different roles in the prenyl-transferase reaction.

As seen in nucleotidyl transfers, there are many enzyme-catalyzed reversible reactions in which bond formations take place with concomitant liberation of inorganic pyrophosphate (PPi). In general, intracellular hydrolysis by pyrophosphatase of the inorganic pyrophosphate thus liberated brings forth a thermodynamic driving force in the direction of bond formation.

The prenyltransferase reaction, however, proceeds with the release of inorganic pyrophosphate in spite of the irreversibility of the reaction (1). This has led us to be interested in the significance of the diphosphate moiety of the substrates of prenyltransferase. In our previous studies with allylic methylenediphosphonates, we showed that the replacement of the

Abbreviations: PPi, inorganic pyrophosphate; -POP, diphosphate moiety; -PCP, methylenediphosphonate moiety; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GPCP, geranyl methylenediphosphonate; IPP, isopentenyl diphosphate; IPCP, isopentenyl methylenediphosphonate; FPP, farnesyl diphosphate.

0006-291X/88 $1.50



Scheme 1.

diphosphate (-POP) moiety of dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) by a methylenediphosphonate (-PCP) moiety resulted in a marked decrease of V_{max} without significant change of Km (2). This fact was explained as a reflection of the poor leaving nature of the methylenediphosphonate group, which is similar in shape to the diphosphate group (Scheme 1) (2-5). This led us to expect that the -POP linkage of isopentenyl diphosphate (IPP) could be replaced by a -PCP linkage without marked loss of reactivity, because PPi is released from allylic substrates but not from IPP.

This paper, however, reports unexpected behavior of isopentenyl methylenediphosphonate (IPCP) in the reaction catalyzed by farnesyl diphosphate (FPP) synthase.

EXPERIMENTAL PROCEDURES

MATERIALS

[1-^{14}C]Isopentenyl diphosphate was a product of Amersham. Nonlabeled allylic diphosphates and [1-^{3}H]Isopentenyl methylenediphosphonate were synthesized essentially according to the method of Davission *et al.*(6) except that tris(tetra-*n*-butyl) ammonium hydrogen methylenediphosphonate was employed instead of tris(tetra-*n*-butyl)ammonium hydrogen diphosphate. [1-^{3}H] Isopentenol (3-methyl-3-butenol) was prepared by reduction of methyl 3-methyl-3-butenoate with sodium borotritiride (specific activity 359 mCi/mmol) (Amersham) in the presence of ethanedithiol (7).

Enzyme Reaction

Farnesyl diphosphate synthase was partially purified from pig liver as described previously (8). The enzyme reaction was followed by determination of the amount of [1-^{14}C]IPP or [1-^{3}H] IPCP incorporated into acid-labile materials. In a standard experiment with [1-^{3}H]IPCP as the homoallylic substrate, the incubation mixture contained, in a final volume of 1.0 ml, 20 μmol of Tris-maleate buffer (pH 6.6), 5 μmol of $MgCl_2$, 10 μmol of 2-mercaptoethanol, 100 nmol of [1-^{3}H]IPCP (spec, act. 0.45 Ci/mol), 25 nmol of GPP and 3.5 units of pig liver FPP synthase. The mixture was incubated at 37 C for 1.5 h, and then made acidic by addition of 0.3 ml of 1 M hydrochloric acid. The radioactive alcohols liberated by acid treatment were extracted

with hexane after the mixture was made alkaline. The radioactivity in the hexane extracts was counted with a liquid scintillation counter.

RESULT

The homoallylic methylenediphosphonate, IPCP was found to be active as substrate for FPP synthase. Kinetic parameters of IPCP are shown in Table 1 along with natural substrates and an allylic analog, geranyl methylenediphosphonate (GPCP). Contrary to our expectation, the V_{max} value of IPCP was only 5.4% of that of IPP. The Km value of IPCP was not so small as that of IPP or GPCP.

In addition, marked differences were observed between IPCP and GPCP with respect to the effects of pH and metal ion concentration. The optimal pH of the reaction of IPCP was around 6.6, whereas that of GPCP was 8.2. Figure 1 shows the effect of magnesium ion concentration on the rate of IPCP reaction at pH 6.6 and 8.2. At pH 6.6 the IPCP reaction showed a hyperbolic curve as a function of magnesium chloride concentration, but at pH 8.2 it showed a maximum at about 0.5 mM of magnesium chloride and declined markedly as the magnesium ion concentration was increased.

IPCP inhibited the enzymatic reaction of natural substrates. Figure 2 shows two sets of double-reciprocal plots of initial velocities for the condensation of IPP and DMAPP. IPCP is a competitive inhibitor against DMAPP with Ki of 0.56 mM, but it is an uncompetitive inhibitor against IPP with Ki of 0.48 mM.

DISCUSSION

Divalent cations such as Mg^{2+} and Mn^{2+} are essential for the prenyltransferase reaction. It is assumed that substrates complexed with metal ions are true substrates for the enzyme. It

Table 1. Kinetic parameters of IPCP in FPP synthase reaction

	IPP	IPCP	GPP*	GPCP*
Km/μM	7.1	36.0	8.1	2.6
V_{max}/nmol min^{-1} mg^{-1}	44.3	2.4	44.0	0.6

* The data are those of our previous study (2).

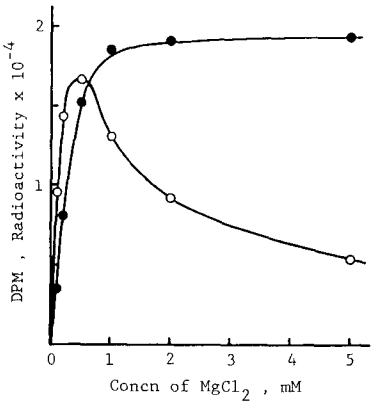


Figure 1. Effect of concentration of magnesium ion on the enzymatic reaction of IPCP at pH 6.6 (●) and pH 8.2 (○).

has been reported that methylenediphosphonic acid, compared with pyrophosphoric acid, has larger pKa values and a stronger affinity for metal ions (4). The reactivity of GPCP, reflecting such characters of the methylenediphosphonate moiety, support the above assumption (2,3).

The results with IPCP, however, are not consistent with the assumption. The reaction with IPCP showed an optimum pH at about 6.6, which was more acidic by 1.1 and 1.4 units than those for the natural substrate and GPCP, respectively. An acidic pH

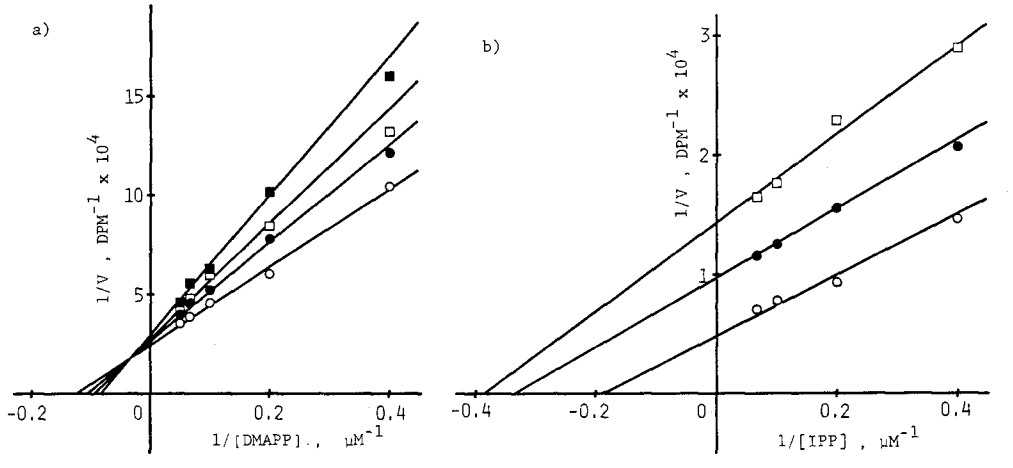
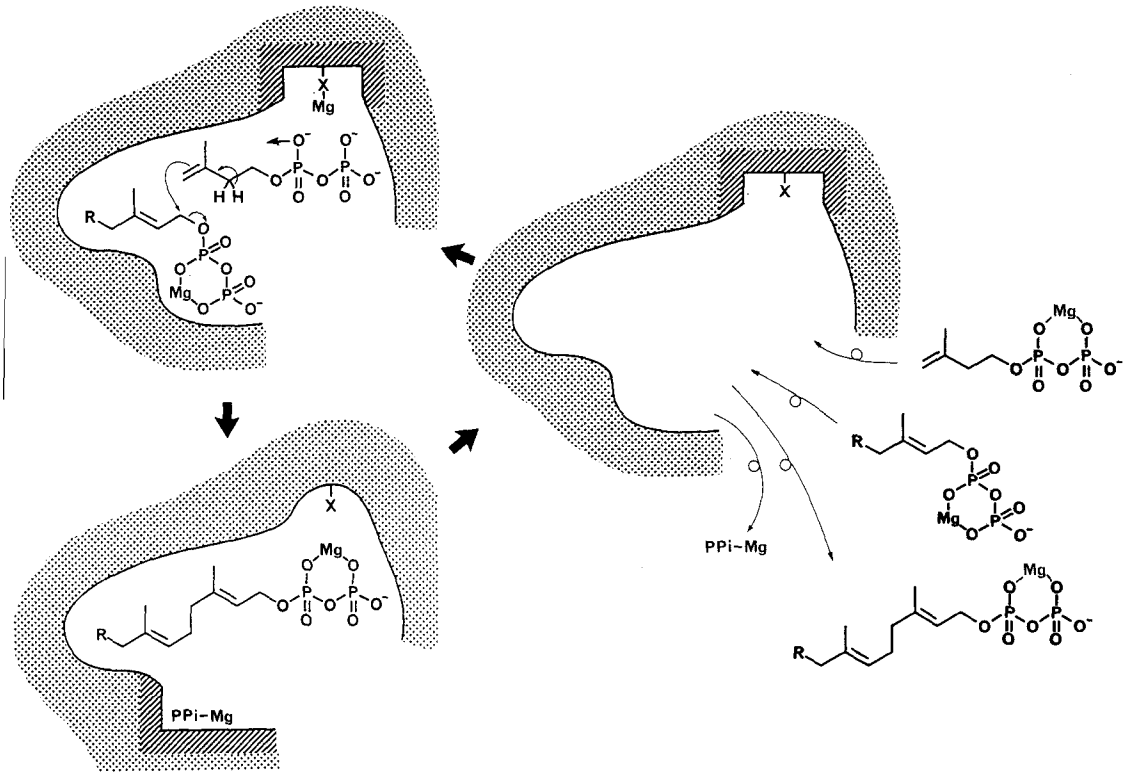


Figure 2. Inhibitory effect of IPCP against DMAPP or IPP. a) The varied substrate was DMAPP. IPCP concentration; 0 (○), 0.1 (●), 0.2 (□), 0.5 mM (■). b) The varied substrate and the allylic substrate were IPP and GPP, respectively. IPCP concentration; 0 (○), 2.0 (●), 3.5 mM (□).

should be disadvantageous to the substrates in forming metal complexes. In addition, the reactivity of IPCP at pH 8.2 was strongly inhibited when the concentration of magnesium ion was raised, whereas such inhibition was not observed at pH 6.6 (Figure 1). These unexpected properties of IPCP against pH and metal ion concentration can not be well interpreted without assuming that the homoallylic substrate, IPP, does react in the metal-free form. Since all the substrate molecules should take the metal complexed forms in the presence of a large excess of magnesium ions, it is necessary to assume a mechanism that involves the capture by the enzyme of the metal ion from the IPP-metal complex. Thus, the magnesium of the IPP-Mg complex is captured by the IPP binding site of the enzyme, and then transferred again to the newly formed allylic diphosphate, which is the product of condensation between the allylic diphosphate complexed with Mg^{2+} and the IPP molecule free of Mg^{2+}. During the condensation reaction, the enzyme may dynamically interact with the substrates and the products including inorganic pyrophosphate to attain an efficient turnover of catalysis. For example, the inorganic pyrophosphate liberated may bind to the enzyme, causing a conformational change of the enzyme in such a way that the captured magnesium is released (Scheme 2). An activation by a small amount of inorganic pyrophosphate was actually observed at an initial stage of the enzymatic reaction (date not shown). Based on the above mechanism, it is explainable that the reaction of IPCP, which has a stronger affinity for metal ions than the natural POP substrate, is strongly inhibited by high concentrations of metal ions. It is likely that the essential role of metal ions in the prenyl-transferase reaction is to form the complex with allylic diphosphates and also probably with the enzyme itself.

Inhibitory effects of IPCP also confirm the difference in behavior of the diphosphate moieties between allylic and homoallylic substrates against metal ion. IPCP was a competitive inhibitor against an allylic substrate, DMAPP, but was an uncompetitive inhibitor against IPP.

Why should the diphosphate moieties of allylic and homoallylic substrates behave so differently with regard to the metal complex formation in the enzymatic reaction ? The complex formation is favorable for the allylic substrate because it accelerates the release of PPi to form the carbonium ion, but it



Scheme 2.

seems unnecessary for IPP to be complexed with Mg^{2+} because PPi is not released from IPP during the condensation. It must be rather advantageous for the IPP to be free of Mg^{2+}, because the negatively charged diphosphate moiety can assist the nucleophilic attack of IPP to the carbonium ion formed from the allylic substrate. It may be the reason why nature uses the diphosphate moiety in prenyltransferase reactions that this moiety can play such dual functions.

ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, the Asahi Glass Foundation for the contribution to industrial technology, and special coordination funds of the science and technology agency of the Japanese Government.

REFERENCES

1. Poulter, C.D. and Rilling, H.C. (1981) In Biosynthesis of Isoprenoid Compounds (J.W.Porter and S.L.Spurgeon, Eds) Vol.1, pp.161-224.

2. Gotoh, T., Koyama, T., and Ogura, K., *Chem. Lett.* **1987**, 1627-1630.
3. Stremler K.E. and Poulter, C.D. (1987) *J. Am. Chem. Soc.* **109**.5542-5544.
4. Irani, R.R. and Moedritzen, K. (1962) *J. Phys. Chem.* **66**,1349-1353.
5. Larsen, M.L., Willett, R., and Yount, R.G. (1969) *Science* **166**, 1510-1511.
6. Davisson, V.Jo, Woodside, A.B., and Poulter, C.D. (1985) In Methods Enzymol. (J.H.Law and H.C.Rilling, Eds) **110**, 130-144.
7. Maki, Y., Kikuchi, K., Sugiyama, H., and Seto, S. (1975) *Tetrahedron Lett.* **38**, 3295-3296.
8. Koyama, T., Saito, Y., Ogura, K., and Seto, S. (1977) *J. Biochem.* **82**, 1585-1590.