MECHANISM AND REGULATION OF THIAMINE PYROPHOSPHOKINASE FROM PARSELY LEAF

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Summary Thiamine pyrophosphokinase (EC 2.7.6.2) from parsely leaf showed an absolute requirement for divalent cation such as Mg²⁺, Mn²⁺ and Co²⁺. The activation effect varied with the species and concentrations of such cations. When Mn²⁺ or Co²⁺ was used as cofactor, maximal activation was found at a lower level than ATP concentration, whereas the activation by Mg²⁺ increased hyperbolically with the concentration. Studies of initial velocity and product inhibition led to conclude that the kinase reaction obeys a sequential ordered Bi Bi mechanism; i.e. the enzyme combines in turn with MgATP and thiamine, followed by release of TPP and AMP. The inhibition type revealed for inorganic pyrophosphate was competitive with respect to thiamine with $K_i$ of approximately 2.8 mM. On the other hand, thiamine monophosphate exhibited noncompetitive inhibition with $K_i$ of 0.2 mM. The plots of the reaction rate against MgATP concentrations gave a sigmoidal curve. Addition of either AMP or GMP resulted in restoration of a depressed activity at low concentration of MgATP. The "allosteric" inhibition was also relieved by the addition of an excess amount of magnesium ions. These findings suggest that transphosphorylation is regulated by subcellular concentrations of metal ions relative to ATP or of the products involved in the thiamine biosynthesis.

Thiamine pyrophosphate (TPP) is known to function as coenzyme in pyruvate (or α-ketoglutarate) dehydrogenase, decarboxylase, transketolase and other enzymes. Thiamine pyrophosphokinase responsible for the TPP synthesis has been found in microorganisms and animal tissues (1, 2). Inadequate purification of the enzyme, until the present authors' studies, hampered in obtaining accurate information concerning enzymatic properties and its kinetics.

A highly purified preparation of thiamine pyrophosphokinase has been

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obtained from parsely leaf (3). By the use of this enzyme preparation, evidence is forwarded for that the parsely enzyme catalyzes the transfer of pyrophosphate moiety from ATP to thiamine but not to TMP (3, 4).

Michaelis constant found for thiamine was at the order of $10^{-7}$ M, indicating that thiamine at subcellular concentration is highly susceptible to the kinase reaction. On the other hand, the Michaelis constant for ATP was of the order of $10^{-3}$ M. The sum of ATP, ADP and AMP concentrations in the aqueous phase of various types of intact cells is between 2 and 15 mM (5). The concentration of ATP usually greatly exceeds the levels of the other two. Because of a high concentration of Mg$^{++}$ in the intracellular fluid, ATP exists primarily as the magnesium complex, which participates as phosphate donor in many enzymatic reactions.

These considerations allow to speculate that the TPP synthesis is favored under intracellular conditions. This follows that if TPP catabolism proceeded at an insignificant rate, pyrophosphorylation of thiamine, a storage form in plant tissues, might cause an abnormal accumulation of TPP. Notwithstanding this hypothetic inference, the intracellular concentration of TPP is in fact kept constant in green leaves of most plants.

The present paper describes the kinetics and regulating mechanisms of thiamine pyrophosphokinase, and provides some explanation for the discrepancy.

MATERIALS AND METHODS

Chemicals. Thiamine and its diphosphate (TPP) were obtained from Sigma Chemical Co. Thiamine monophosphate (TMP) was kindly supplied by Dr. S. Takei, Research and Developing Division of Takeda Chemical Industries Co., Ltd. ATP, AMP and GMP were products of Kojin Co., Ltd. Other chemicals were of the highest grade commercially available.

Enzyme assays. Thiamine pyrophosphokinase was purified to electrophoretic homogeneity from parsely leaves in the same manner as reported in the preceding paper (3). The enzyme reaction was carried out at 37°C for 60 min in 0.05 M Tris-HCl buffer of pH 8.0. The amount of TPP formed was determined as described in the previous paper (4) by the manometric measurement of the CO$_2$ evolved from pyruvate with a large excess of apopyruvate decarboxylase in a Warburg apparatus. For kinetic analysis, the concentration of enzyme was adjusted so that the conversion of substrate did not exceed 20% of the total under the standard assay conditions. The reaction rate at early stages was represented in terms of volume ($\mu$l) of carbon dioxide evolved per 30 min.

RESULTS

Divalent cation requirement

An absolute requirement of magnesium ion for activity of thiamine pyrophos-
phokinase was replaced to some extent by manganese and cobalt ions among a number of divalent cations tested, as reported in the previous paper (3). Figure 1 compares activation by varied concentrations of Mg$^{2+}$, Mn$^{2+}$ and Co$^{2+}$. In a separate series of experiments it was ascertained that the pH dependence of the enzymatic reaction in the presence of either Mn$^{2+}$ or Co$^{2+}$ was similar to that observed in the presence of Mg$^{2+}$. In the presence of 2 mM ATP, a maximal activation was attained with 0.5 mM Mn$^{2+}$, 1 mM Co$^{2+}$, or with a concentration of Mg$^{2+}$ higher than 3 mM. Both manganese and cobalt ions at higher concentrations showed rather inhibitory effect on the enzyme activity, while a hyperbolic activity-profile was obtained with increasing concentrations of magnesium ions. The magnesium concentration sufficient to produce maximal activation varied in proportion to the ATP concentration. This saturation effect is compatible with the notion that an active 1:1 complex is formed between ATP and Mg$^{2+}$. The other two cations may also be regarded as forming complex with ATP to give an active pyrophosphate donor. The explanation of the difference in the saturation effect between magnesium and cobalt (or manganese) concentrations remains obscure.

**Fig. 1.** Effect of increasing concentrations of divalent cations on thiamine pyrophosphokinase activity. The reaction mixture contained, besides divalent cations at indicated levels, as follows: Tris amino-methane (pH 8 with HCl), 0.05 M; ATP, 2.0 mM; thiamine, 5.0 $\mu$M; enzyme protein, 100 $\mu$g/ml. The mixture was incubated at 37°C for 60 min. The assay procedures were described in the text and in the preceding paper (3).

**Bireactant initial velocity pattern**

Variation of the concentration of one substrate at different fixed levels of the another one allows to determine the initial velocity pattern of the enzymic reaction involving two substrates. Figure 2 shows such a graphical determination with thiamine pyrophosphokinase. A series of straight lines crossing on the horizontal axis was obtained from the double reciprocal plots of the reaction rate against varying thiamine concentrations at constant MgATP levels (left in Fig. 2), *vice versa*
Fig. 2. Double reciprocal plots of initial velocity vs. varied concentrations of one substrate at fixed levels of the other substrate for thiamine pyrophosphokinase reaction. The reaction was carried out at 37°C for 60 min with 200 μg of enzyme protein in 2 ml of 0.05 M Tris-HCl buffer (pH 8). In the left, MgATP concentration was fixed at 1.0 (A), 1.5 (B), 2.0 (C) and 4.0 mM (D), respectively; in the right, thiamine concentration was at 0.3 (A), 0.5 (B), 1.5 (C) and 3.0 μM (D), respectively.

(right in Fig. 2). This fact suggests that the reaction obeys a "sequential" mechanism as defined by Cleland (6) and that both substrates must combine with the enzyme before release of either product, the mechanism being apparently different from a ping-pong mechanism. The intercepts of the series of straight lines in Fig. 2 gave the Km values of 2.9 μM for thiamine and of 4.5 mM for equimolar complex of ATP and Mg2+, both of which are significantly higher than those determined in the presence of an excess Mg2+ (3).

Product inhibition by TPP and AMP

According to the Cleland’s classification (6), a sequential Bi Bi mechanism is further divided into two categories; i.e. ordered and random mechanisms. Initial velocity studies make no distinction between them. Since TPP functioned as a potent inhibitor, the inhibition pattern was detailed in order to identify the order of combination and release of reactants. In Figs. 3 and 4 are established the type of product inhibition with TPP at varied concentrations of one substrate and at constant levels (unsaturated) of the other substrate. The inhibition pattern was noncompetitive with respect to both variable substrates, thiamine and MgATP, with Ki values of 0.55 μM and 0.65 μM respectively.

In Fig. 5 is analyzed the type of AMP inhibition with thiamine as variable substrate and at unsaturated level of MgATP. The type of inhibition was again non-competitive and the Ki was approximately 37 mM. The noncompetitive inhibition by AMP, however, was extinguished by saturation with MgATP (more than 20 mM).
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Fig. 3. Product inhibition by TPP with thiamine as variable substrate and MgATP as fixed substrate. The assay conditions were similar to those described in Fig. 2 (left), except for 2 mM MgATP and TPP of varied levels; A, 0.25 μM; B, 0.125 μM; C, none added.

Fig. 4. Product inhibition by TPP with MgATP as variable substrate and thiamine as fixed substrate. The assay conditions were the same as shown in Fig. 2 (right), except for 1.5 μM thiamine and TPP of varied levels; (A) 0.5 μM, (B) 0.25 μM and (C) none added.

Figure 6 depicts the double reciprocal plots for the determination of AMP inhibition with ATP as variable substrate and thiamine as fixed one. In this case, a large excess amount of Mg^{2+} was added to the assay system, to avoid substrate inhibition by free ATP which will be described later. In Fig. 6, a series of lines intersect at the vertical axis indicating that AMP at high concentrations competes with MgATP on the same active site of the enzyme. It was evidenced from these findings that combination of MgATP and thiamine was ordered with concomitant release of TPP and AMP in turn.

Inhibition by PP<sub>1</sub> and TMP

PP<sub>1</sub> and TMP were the most potent inhibitors among various compounds.
Fig. 5. Product inhibition by AMP at fixed level of MgATP. The assay conditions were the same as indicated in the left of Fig. 2, except for 3 mM MgATP. The concentrations of AMP added were as follows; A, 100 mM; B, 50 mM; C, none added.

Fig. 6. Product inhibition by AMP with ATP as variable substrate and with magnesium ion and thiamine at fixed high levels. The assay conditions were similar to those in the right of Fig. 2, except for 100 μM thiamine and 10 mM magnesium ion. The concentrations of AMP added were as follows; A, 100 mM; B, 50 mM; C, 20 mM; D, none added.

Fig. 7. Competitive inhibition by inorganic pyrophosphate. The experimental conditions were the same as described in Fig. 2 (for the left), except for 20 mM MgATP. The concentrations of PPi added were as follows; (A) 4 mM, (B) 2 mM, (C) 1 mM and (D) none added.

Fig. 8. Noncompetitive inhibition by thiamine monophosphate. The assay conditions were the same as in Fig. 2 (for the left), except for 20 mM MgATP and TMP at varied concentrations; (A) 200 μM, (B) 100 μM and (C) none added.
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tested (3). Figures 7 and 8 show graphical determinations of the inhibition patterns and constants for these inhibitors with respect to thiamine as variable substrate at higher level of MgATP (20 mM). The double reciprocal plots appear to the linear within experimental errors. In Fig. 7, a series of straight lines intersect at the vertical axis, demonstrating that the type of PPi inhibition is competitive with the $K_i$ value of about 2.8 mM. The inhibition by PPi proved to be non-linear and competitive with respect to MgATP as variable substrate at a higher level of thiamine. On the other hand, the plots for TMP inhibition intersected on the horizontal axis as shown in Fig. 8, exhibiting a typical pattern for non-competitive inhibition with the inhibition constant of 0.2 mM. It thus follows that TMP possibly interferes with combination of thiamine in a similar fashion as was found with to TPP.

*Regulation of the kinase activity by ATP*

Figure 9 illustrates the variations of the reaction rate with varying metal ion and ATP concentrations at a constant ratio. Since the dissociation constant found for MgATP complex is around $14 \mu M$ at pH 8, the complex form predominates in an equimolar mixture of ATP and Mg$^{2+}$ within the concentration range employed in Fig. 9. Nevertheless, the curve depicted in Fig. 9 (open circles) shows a typical sigmoidal nature. Addition of a fixed but excess amount of magnesium over

![Fig. 9](image)

**Fig. 9.** Relationship between MgATP concentrations and initial velocity. The initial velocity was expressed as nmoles of TPP formed per hr under the same assay conditions as described in Fig. 2, except for 100 $\mu$M thiamine. Symbols denote additions to the assay medium: $\bullet$—$\bullet$, 10 mM Mg$^{2+}$; $\circ$—$\circ$, 5 mM AMP or GMP; $\bigcirc$—$\bigcirc$, control.

![Fig. 10](image)

**Fig. 10.** Hill plots of data shown in Fig. 9 (open circles).
ATP resulted in restoration of the activity at lower concentrations of the substrate. This fact implies that the free ATP behaves as an inhibitor rather than as an inert substrate. AMP and GMP which had behaved as poor inhibitors in the assay medium containing an excess magnesium, also could eliminate the sigmoidal nature. Solid circles in Fig. 9 represent the experimental results. The experimental data shown in Fig. 9, open circles, were further plotted in Fig. 10 according to the Hill equation (7). The Hill coefficient of 1.8 was calculated from the plots, indicating that the binding site for free ATP might possibly be different from that of the metal complex acting as pyrophosphate donor. This possibility is supported by the fact that "allosteric" inhibition is restored by adding AMP or GMP which shows little or no ability in complex formation. Observed "allosteric" inhibition is presumably involved as an regulatory mechanism in TPP biosynthesis in vivo. Details of the inhibition by ATP remained to be further investigated.

DISCUSSION

Kinetic studies of initial velocity and product inhibition patterns led to conclude that the reaction of thiamine pyrophosphokinase obeys a sequential ordered Bi Bi mechanism in a fashion resembled to those of mevalonic kinase (8), hexokinase (9) and creatine kinase (10). The conclusion obtained in this paper, however, incompatible with that of THOMÉ-BEAU et al. (11) who postulated a ping-pong mechanism for the combination of substrate with the yeast enzyme. If the ping-pong mechanism is operative, the ratio of $K_m$ to $V_{max}$ must be constant irrespective of the concentration of fixed substrate. The present authors' data shown in Fig. 2 are in clear contradict with this relationship. This marked discrepancy may be attributable to the difference in the enzyme source and its purities: the enzyme they used is from yeast, and a partially purified preparation and may be contaminated with other active proteins, while thiamine pyrophosphokinase from parsely employed in the present paper is electrophoretically homogeneous on polyacrylamide gel (3). A question arises as to their double reciprocal plots which gave a series of lines being parallel to each others and also to the horizontal axis at varied concentrations of fixed substrate. In addition, product inhibition was outside of the scope of the paper presented by THOMÉ-BEAU et al. Product inhibition studied in the present paper lends support for a sequential ordered Bi Bi mechanism;

![Fig. 11. A diagram of ordered Bi Bi mechanism.](image-url)
Thiamine pyrophosphokinase from parsley exhibits an extremely high affinity for thiamine ($K_m$ value is of the order of $10^{-7}$ M). Judging from the level of MgATP complex in cell fluids, this high affinity allows to predict that an over-production of TPP is favored under the intracellular conditions. However, the intracellular concentration of TPP is in fact kept less than the levels of a storage-form of thiamine in most plant green leaves. The fact indicates that a certain regulation mechanism is involved in the TPP metabolism in vivo. The pattern of TPP inhibition revealed in Figs. 3 and 4 was noncompetitive and its constant was of the order of $\mu$M, the orders being approximate to the intracellular level of TPP in plants. This product inhibition implies that TPP may play a role, at least in part, in controlling its own synthesis in vivo.

The reaction of thiamine pyrophosphokinase with two substrates is susceptible to the inhibition by ATP added at a level higher than magnesium concentration, but not by another substrate thiamine added even at much higher concentrations. The uncomplexed ATP has been well-known not to function as active substrate but rather behave as inhibitor in a number of enzyme reactions (12-14). For example, phosphofructokinase (EC 2.7.1.11) exhibits “sigmoidal” kinetics when the enzyme activity is measured as a function of fructose-6-phosphate concentration at higher levels of ATP (15). On the other hand, the kinetics become “hyperbolic” at a fixed but low ATP concentration. Such a sigmoidal curve was also observed in the reaction of thiamine pyrophosphokinase with MgATP as variable substrate, whereas the curve became “hyperbolic” in the presence of a fixed but excess AMP, GMP or Mg$^{2+}$ over ATP concentration (Fig. 9). This phenomena can be interpreted in terms of a function of ATP occupying a regulatory site which is distinct from the active site; that is, AMP or GMP modulates the TPP synthesis by competing with ATP on a site of regulation and Mg$^{2+}$ also controls the activity of thiamine pyrophosphokinase by forming chelate with ATP. In addition to these regulation mechanisms, the activity of thiamine pyrophosphokinase is the subject of inhibition

![Diagram](image-url)

Fig. 12. Thiamine pyrophosphate synthesis cycle and its regulation in plants.
by TMP and PP, which are the direct products from OMP-PP and Th-P in the sequence of overall TPP biosynthesis. These findings, taken together with the interpretations made and inferences drawn in the present and previous papers, allow to propose a biosynthesis cycle for TPP and its regulation such as depicted diagramatically in Fig. 12.

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