BIOGENESIS OF COCARBOXYLASE IN ESCHERICHIA COLI : A NOVEL ENZYME CATALYZING THE FORMATION OF THIAMINE PYROPHOSPHATE FROM THIAMINE MONOPHOSPHATE

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SUMMARY

An enzyme catalyzing the formation of thiamine pyrophosphate from thiamine monophosphate in the presence of ATP and Mg^{++} has been found in the soluble fraction of Escherichia coli K12. The enzyme was markedly stimulated by either potassium or ammonium ion. The physiological significance of the enzyme in the biogenesis of cocarboxylase in E. coli is discussed.

It has been established that thiamine pyrophosphate (TPP, cocarboxylase) is formed by a direct transfer of a pyrophosphate group from ATP to thiamine in microbiol and mammalian systems (1,2). The enzyme responsible has been termed thiamine kinase (ATP : thiamine pyrophosphotransferase, EC 2.7.6.2.), which has been demonstrated in baker's yeast (1), liver (2) and the membrane fraction of Escherichia coli (3).

On the other hand the pathways of thiamine biosynthesis in baker's yeast (4-7) and E. coli (8) from hydroxymethylpyrimidine and hydroxyethylthiazole has been clarified (Fig. 1.), and thiamine monophosphate (TMP) was found to be the product formed enzymatically from these two moieties of thiamine. Since TMP can not be used as substrate for purified yeast thiamine kinase (1), it has been supposed that TMP must be dephosphorylated to thiamine before it can be converted to TPP (9).

In the present paper, evidence is presented that an enzyme, which

Hydroxymethylpyrimidine $\xrightarrow{ATP}$ Hydroxymethylpyrimidine monophosphate

Hydroxymethylpyrimidine monophosphate $\xrightarrow{ATP}$ Hydroxymethylpyrimidine pyrophosphate

Hydroxyethylthiazole $\xrightarrow{ATP}$ Hydroxyethylthiazole monophosphate

Hydroxymethylpyrimidine pyrophosphate + Hydroxyethylthiazole monophosphate $\rightleftharpoons$ Thiamine monophosphate + PP

Fig. 1. The pathways of thiamine biosynthesis in yeast and E. coli.

catalyzes a direct phosphorylation of TMP to TPP in the presence of ATP and Mg^{++}, namely thiamine monophosphate kinase, exsists in the soluble fraction of E. coli K12.

MATERIALS AND METHODS

The disodium salt of ATP and thiamine pyrophosphate chloride were purchased from Sigma Chemical Company. Thiamine monophosphate chloride was kindly supplied by Dr. S. Yurugi of Takeda Chemical Industries, Osaka, Japan.

The cells of a E. coli K12 were grown in the minimal medium of Davis and Mingioli (10). They were harvested at the stationary phase and washed once with a saline solution, and then suspended in a solution of 0.05 M Tris-HCl, pH 7.5, 2mM 2-mercaptoethanol and 1mM EDTA. The cell suspension was treated for 10 min at 4° in a sonic oscillator (10 KC), then centrifuged for 20 min at 15,000 x g, and the supernatant fluid was used as a crude extract. The crude extract was treated with one tenth volume of 2 % protamine sulfate solution. After standing for 5 min at 0°, the precipitate was collected by centrifugation at 15,000 x g for 20 min.

The pellet was stirred with 0.05 M K-phosphate buffer, pH 7.5, containing 0.5 M KCl, 2mM 2-mercaptoethanol and 1mM EDTA for 20 min. The suspension was

centrifuged at 15,000 x g for 20 min, and the supernatant fluid was collected. The protamine eluate was then treated by ammonium sulfate fractionation between 20-40 % saturation. The precipitate was dissolved in a small volume of 0.05 M K-phosphate buffer, pH 7.5, 2mM 2-mercaptoethanol and 1mM EDTA.

Thiamine monophosphate kinase activity was meausured as follows. The reaction mixture contained : 100 μ moles Tris-HCl, pH 7.5 ; 10 μ moles ATP ; 10 μ moles $MgCl_2$; 1 mmole KCl, 30 mμ moles TMP ; and the enzyme preparation (1-3 mg protein) in a total volume of 3 ml. After incubation for 30 min at 37°, the reaction was stopped by heating for 5 min at 90°, followed by centrifugation to remove denatured protein.

TPP in the supernatant fluid was determined manometrically by carbon dioxide evolution with yeast apocarboxylase and sodium pyruvate (11).

The reaction product was identified by bioautographic techniques. Deproteinized reaction mixture was lyophilized and dissolved in 0.3 ml of

TABLE I

Effect of K^+ and NH_4^+ on thiamine monophosphate kinase

Additions (M)	TPP formed (mμ moles/mg/30 min)
Complete	0.002
" + K^+, 0.17	0.049
" + K^+, 0.33	0.070
" + K^+, 0.66	0.043
" + NH_4^+, 0.33	0.071

Complete system, in a volume of 3 ml, contained : 100 μ moles Tris-HCl, pH 7.5, 10 μ moles ATP, 10 μ moles $MgCl_2$, 30 mμ moles TMP, and the enzyme preparation (crude extract, 2.2 mg protein). K^+ was present as KCl, and NH_4^+ as $(NH_4)_2SO_4$.

distilled water. Two microliters of the concentrated sample was spotted on Toyo filter paper (No. 50, 2 X 40 cm) and developed by ascending method in a solvent system ; isopropyl alcohol-0.5 M acetate buffer, pH 4.5-H_2O, 65 : 15 : 20, V/V. The developed paper chromatogram was applied to bioautography using E. coli thiamineless (strain 70-23), as previously described (7).

RESULTS

As shown in Table I, thiamine monophosphate kinase activity was found in the crude extract of E. coli K12 and it was markedly stimulated by the addition of K^+ and NH_4^+. Na^+ had only a limited capacity to replace K^+. In the presence of 0.33 M KCl the activity was proportional to the amounts of enzyme added, up to 3 mg protein, and the reaction preceeded linearly for 60 min when 3 mg enzyme protein was used.

TABLE II

Requirements for thiamine monophosphate kinase reaction

Additions	TPP formed (mμ moles/mg/30 min)
Complete	0.762
" - TMP	0.023
" - ATP	0.010
" - Mg^{++}	0.014
" - K^+	0.478
" - TMP + thiamine	0
" but boiled enzyme	0.020

Complete system, in a volume of 3 ml, contained : 100 μ moles Tris-HCl, pH 7.5, 10 μ moles ATP, 10 μ moles $MgCl_2$, 1 mmole KCl, 30 mμ moles TMP, and the enzyme preparation (20-40 % ammonium sulfate fraction, 1.7 mg protein).

Table II shows requirements for the reaction catalyzed by a partially purified enzyme. The maximal rate of reaction was dependent on the presence of TMP, ATP, Mg^{++}, and the enzyme. The effect of omission of K^{+} was not so remarkable in this experiment, because of the presence of ammonium sulfate in the enzyme preparation used. No activity was found with thiamine in place of TMP. This would be against a two step formation of TPP from TMP via thiamine.

The present assay method was fairly specific for TPP, and thus it clearly indicated the reaction product as TPP. Further identification of TPP was carried out by bioautography. As shown in Fig. 2. the reaction product was identified as TPP, whereas thiamine triphosphate (TTP) as well as thiamine was not detected.

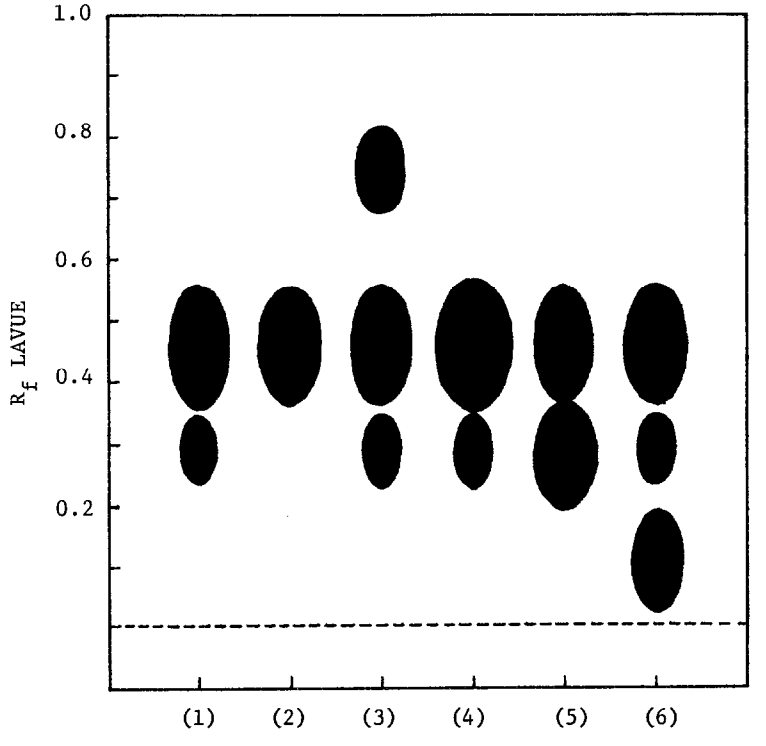

Fig. 2. A drawing of a bioautogram which illustrates the nature of the product by thiamine monophosphate kinase reaction. Aliquots of 2 µl of the concentrated reaction mixture, which was the same as described in the legend of Table II, were chromatographed and bioautographed as described in the text. (1), complete ; (2), complete-ATP ; (3), (4), (5) and (6) were complete, cochromatographed with authentic thiamine, TMP, TPP and TTP in 0.04 mµ mole amounts respectively.

DISCUSSION

The results described above indicate that a novel enzyme, which catalyzes the formation of TPP from TMP by transfer of one phosphate group of ATP, is present in the soluble fraction of E. coli K12. The reasons are as follows. First, only TMP was an effective substrate for a partially purified enzyme, whereas thiamine could not be used as substrate. Secondly, TPP was identified as a product of the reaction and neither TTP nor thiamine was detected on the bioautogram. Thirdly, the reaction was hardly inhibited by pyrithiamine (12), a potent inhibitor of thiamine kinase (1,2).

These data therefore provide evidence that TMP formed by thiamine synthesizing system in E. coli can be converted directly to TPP by this enzyme without making a detour via thiamine. Miyata et al. (3) reported that thiamine kinase is present in the sonicated membrane fraction of E. coli and is located in the spheroplast membrane and that the kinase probably participated in the transport of thiamine. On the other hand the presence of thiamine monophosphate kinase in the soluble fraction of E. coli K12, in which thiamine synthesizing enzymes are known to be located (13), is quite reasonable for biogenesis of cocarboxylase in E. coli.

Further attempt to purify this enzyme is now in progress.

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