BBA Report

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Conversion of thiamine to thiamine monophosphate by cell-free extracts of *Escherichia coli*

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SUMMARY

Enzymatic synthesis of thiamine monophosphate from thiamine in the presence of ATP and Mg^{2+} by cell-free extracts of *Escherichia coli* K 12 was demonstrated. The enzyme was markedly stimulated by either K^+ or NH_4^+, and the apparent K_m for thiamine was $2.8 \cdot 10^{-6}$ M.

It has been established that thiamine monophosphate can be formed either by enzymatic synthesis from hydroxymethylpyrimidine pyrophosphate and thiazole monophosphate[1,2] or by enzymatic degradation of thiamine pyrophosphate[3] in microorganisms, but there has been no well-documented evidence that thiamine monophosphate can be synthesized by the phosphorylation of free thiamine[4].

Recently, however, Hayashi and Nakayama[5] found that thiamine added to an external medium is accumulated as thiamine monophosphate in the cells of a thiamine pyrophosphate-less mutant of *Escherichia coli* W, and suggested the formation of thiamine monophosphate from thiamine in this mutant.

In the present report, evidence is presented that a new enzyme, which catalyzes the phosphorylation of thiamine to thiamine monophosphate in the presence of ATP and Mg^{2+}, namely thiamine kinase, exists in the soluble fraction of *E. coli* K 12.

The cells of a *E. coli* K 12 were grown in the minimal medium of Davis and Mingioli[6]. They were harvested at the stationary phase, washed once with a saline solution, and then suspended in a solution of 0.05 M Tris–HCl, pH 7.5, 2 mM 2-mercaptoethanol and 1 mM EDTA. The cell suspension was treated for 10 min at 4° in a sonic oscillator (10 kcycles/sec), then centrifuged for 20 min at 15 000 x *g*, and the supernatant fluid was used as the cell-free extract.

Thiamine kinase activity was assayed under the conditions given in Table I. As shown in Table I, thiamine kinase activity was found in cell-free extracts of *E. coli* K 12. The phosphorylation of thiamine was dependent on the presence of ATP, Mg^{2+} and the

TABLE I

FORMATION OF THIAMINE MONOPHOSPHATE BY CELL-FREE EXTRACTS OF *E. COLI*

The reaction mixtures contained 20 μmoles of Tris–HCl, pH 7.5, 4 μmoles of ATP, 4 μmoles of $MgCl_2$, 6 nmoles of [^{14}C] thiamine★ and the cell-free extract (1.1 mg protein) in a final volume of 0.6 ml. The mixtures were incubated for 1 h at 37°. After adding 50 μl of 2 M acetate buffer (pH 4.5), the reaction was terminated by heating for 5 min at 90°, followed by centrifugation to remove denatured protein. Aliquots (50 μl) of deproteinized incubation mixtures were chromatographed (ascending) on Toyo filter paper (No. 50, 2 cm x 40 cm) with isopropanol–0.5 M acetate buffer (pH 4.5)–water (65:15:20, by vol.) as the solvent system, and co-chromatographed with authentic thiamine monophosphate. The developed chromatograms were surveyed under ultraviolet light, and the ultraviolet-absorbing spot of thiamine monophosphate was cut out. After drying they were put into 10 ml of Bray's solution, and the radioactivity was measured in a Packard Model 3375 Tri-Carb scintillation spectrometer.

Incubation mixture	*Thiamine monophosphate formed (nmoles/mg per h)*
Complete	0.346
Complete – ATP	0.058
Complete – Mg^{2+}	0.035
Complete + KCl, 0.2 M	1.10
Complete + NH_4Cl, 0.2 M	0.813
Complete but boiled enzyme	0.023

★Thiamine thiazole-2-^{14}C; 25.1 mCi/mmole, the Radiochemical Centre, England.

enzyme, and was markedly stimulated by the addition of K^+ or NH_4^+. The activity was proportional to the amount of enzyme added up to 2 mg protein, and the reaction proceeded linearly for 2 h when 1 mg enzyme protein was used. The reaction followed saturation kinetics, with an apparent K_m value for thiamine of $2.8 \cdot 10^{-6}$ M, as extrapolated from the Lineweaver–Burk plot (Fig. 1).

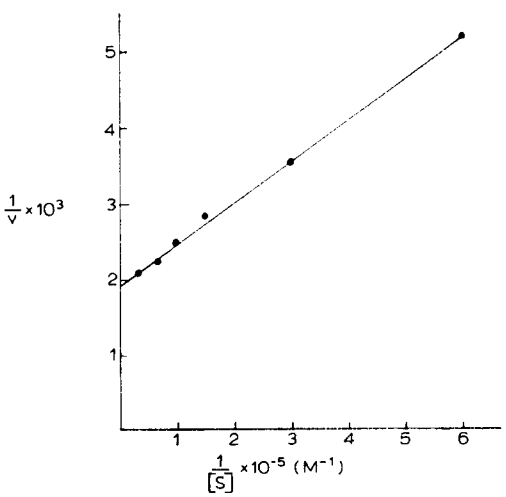

Fig. 1. Lineweaver–Burk plot of the conversion of thiamine to thiamine monophosphate by cell-free extracts of *E. coli*. Incubation mixtures as described in Table I, and containing varying substrate concentrations as shown, were incubated for 1 h at 37°, then inactivated, chromatographed, and assayed as in Table I.

Fig. 2 shows the incorporation by the enzyme of [^{14}C] thiamine into thiamine monophosphate in the presence of ATP and Mg^{2+}. Thiamine monophosphate was identified as a product of the reaction, and more than 95% of the radioactivity of [^{14}C] thiamine, reduced during the incubation, was recovered in the thiamine monophosphate fraction. No thiamine pyrophosphate was formed under the conditions used, which was also confirmed by manometric determination of thiamine pyrophosphate and bioautographic techniques using a thiamine-less mutant of *E. coli* (strain 70-23).

These results would rule out the possibility that thiamine monophosphate was formed as a degradation product of preformed thiamine pyrophosphate. Thus, free thiamine can be converted to thiamine pyrophosphate *via* thiamine monophosphate by two-step phosphorylations by thiamine kinase and thiamine monophosphate kinase, which was also recently demonstrated in cell-free extracts of *E. coli* K 12 (ref. 7), although thiamine pyrophosphokinase (ATP:thiamine pyrophosphotransferase, EC 2.7.6.2) has not been detected in the soluble fraction of *E. coli*[8].

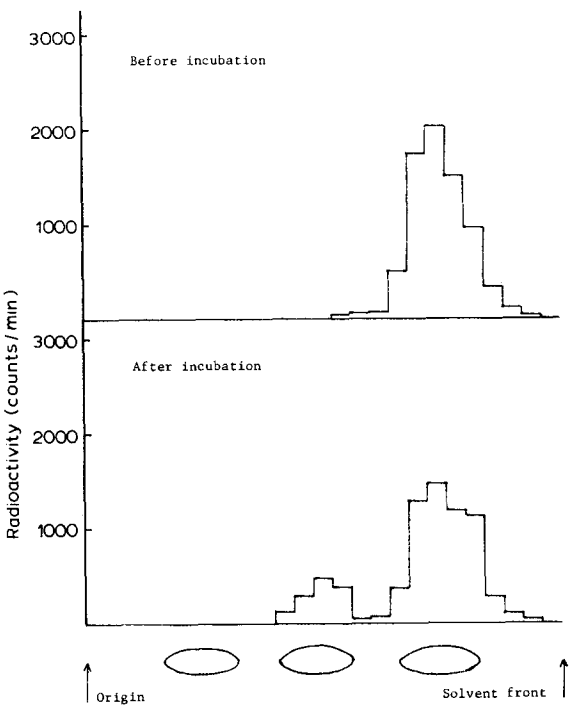

Fig. 2. Incorporation of [^{14}C] thiamine into thiamine monophosphate by cell-free extracts of *E. coli*. Incubation mixtures containing 1.8 mg of enzyme protein, as described in Table I, were incubated for 1 h at 37°, then inactivated. Aliquots (50 μl) were developed with authentic thiamine (R_F, 0.72), thiamine monophosphate (R_F 0.41) and thiamine pyrophosphate (R_F 0.24) in a solvent system as described in Table I. After surveying the localization of thiamine derivatives cochromatographed under ultraviolet light the papers were cut into segments (1 cm in length) and all areas of the paper were quantitatively assayed for radioactivity by the liquid scintillation technique.

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