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THE ROLE OF BOUND THIAMINE PYROPHOSPHATE IN THE SYNTHESIS OF THIAMINE TRIPHOSPHATE IN RAT LIVER

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Summary

Thiamine pyrophosphate-ATP phosphoryltransferase, the enzyme that catalyzes the synthesis of thiamine triphosphate, has been found in the supernatant fraction of rat liver. The substrate for the enzyme is endogenous, bound thiamine pyrophosphate, since the addition of exogenous thiamine pyrophosphate had no effect. Thus, when a rat liver supernatant was incubated with γ-labelled [^{32}P] ATP, thiamine [^{32}P] triphosphate was formed whereas the incubation of thiamine [^{32}P] pyrophosphate with ATP did not produce thiamine [^{32}P]-triphosphate. The endogenous thiamine pyrophosphate was found to be bound to a high molecular weight protein which comes out in the void volume of Sephadex G-75, and is not dialyzable. The activity that catalyzes the formation of thiamine triphosphate has an optimum pH between 6 and 6.5, a linear time course of thiamine triphosphate synthesis up to 30 min, and is not affected by Ca^{2+}, cyclic GMP and sulfhydryl reagents.

Introduction

Thiamine triphosphate has been long known to exist in animal tissues, especially in liver, heart and brain [1]. Of the four forms of the vitamin, about 80% is thiamine pyrophosphate, 5—10% is thiamine triphosphate, and the remainder is thiamine monophosphate and free thiamine [2]. Although the role of thiamine pyrophosphate as a coenzyme in intermediary metabolism is well established, no function of thiamine triphosphate has yet been elucidated: thiamine triphosphate cannot replace thiamine pyrophosphate as a coenzyme nor can it substitute for ATP in kinase reactions [3,4]. However, the role of thiamine triphosphate in subacute necrotizing encephalomyelopathy (Leigh's disease) has been well described. Patients with this neurological, genetic disease elaborate a macromolecule which inhibits the conversion of thiamine pyro-

phosphate to thiamine triphosphate in brain. When these patients die, their brain level of thiamine triphosphate is considerably reduced compared to normal brain [5—8].

At present, little is known about the synthesis of thiamine triphosphate. A thiamine pyrophosphate-ATP phosphoryltransferase activity has been reported to be present in a particulate fraction in rat and bovine brain [9,10]. However, this activity to date has resisted purification or characterization. On the other hand, two species of thiamine triphosphatase have been reported with pH optimum of 6.5 and 9 which catalyze the hydrolysis of thiamine triphosphate into thiamine pyrophosphate and inorganic phosphate [11,12].

In the present study, phosphoryltransferase activity catalyzing the synthesis of thiamine triphosphate from thiamine pyrophosphate and ATP has been isolated from rat liver and shown to utilize endogenously bound thiamine pyrophosphate as substrate.

Materials and Methods

Materials. Thiamine, thiamine pyrophosphate and ATP were obtained from the Sigma Chemical Co. Thiamine triphosphate was a generous gift from Sankyo (Japan). [^{35}S] Thiamine, [γ-^{32}P] ATP, and scintillation fluid (ACS) were obtained from Amersham-Searle. Thiamine [^{32}P] pyrophosphate was synthesized from $H_3{}^{32}PO_4$ and thiamine, and purified as described by Matsukawa et al. [13].

Preparation of rat liver supernatant. All steps in the preparation were carried out at 0—4° C. Fresh rat livers were homogenized in four volumes of 0.25 M sucrose using a glass homogenizer with motor drive teflon pestle. Nuclear and mitochondrial fractions were collected by centrifugation at 750 × *g* and 10 800 × *g*, respectively, for 30 min; the microsomal fraction was prepared by centrifugation at 100 000 × *g* for 90 min. All particulate fractions were resuspended in 0.25 M sucrose. The 100 000 × *g* supernatant fraction retained enzymatic activity at 0—4° C for at least 7 days.

^{35}S-labelled rat liver supernatant. [^{35}S] Thiamine (specific activity 38 Ci/mol) was injected intraperitoneally into thiamine-deficient rats as described by Sen and Cooper [14]. After 24 h the rats were decapitated, and the livers were dissected and homogenized in 0.25 M sucrose. The homogenate was fractionated and the supernatant was prepared as described above. About 80% of the total radioactivity was found in the supernatant fraction.

Assay for thiamine triphosphate-synthesizing activity. The standard assay mixture in total volume of 100 µl contained 50 mM potassium phosphate buffer, pH 6.0, 2.5 mM ATP, 2.5 mM Mg^{2+}, an appropriate amount of [γ-^{32}P]-ATP (final specific activity in the reaction mixture was about 600 cpm/nmol) and 40—60 µl of liver supernatant (40—50 µg protein/µl). Incubations were carried out for 30 min at 37° C and the activity terminated by the addition of 10 µl of 20% trichloroacetic acid. After centrifugation, the trichloroacetic acid was removed by a double extraction with three volumes of water-saturated ether. After the residual ether was evaporated, 20 µl of the solution was subjected to paper electrophoresis in pyridine/acetate buffer, pH 4.6 (20 ml pyridine/8 ml glacial acetic acid in 3 l of water) at a current of 2 mA per strip

for 90 min [15]. Standards of thiamine pyrophosphate and thiamine triphosphate were applied to each strip prior to electrophoresis to locate the bands. The papers were then dried, sprayed with thiochrome reagent [15] and the thiamine, thiamine monophosphate, thiamine pyrophosphate, and thiamine triphosphate bands were located under ultraviolet light. Each strip was cut into pieces, eluted with distilled water and the radioactivity determined after the addition of scintillant. Blank determinations were carried out in the same manner except trichloroacetic acid was added to the incubation mixture before the addition of enzyme.

Protein was determined by the method of Lowry et al. [16] using bovine serum albumin as standard.

Results

As shown in Table I, when subcellular fractions from rat liver were incubated with [γ-^{32}P]ATP, most of the thiamine [^{32}P]triphosphate that was formed was found in the supernatant fraction. Thus, the thiamine triphosphate-synthesizing activity in rat liver is largely localized in the supernatant and not in the particulate fraction as with the rat brain enzyme. It should be noted that the thiamine [^{32}P]triphosphate has been produced from the endogenous thiamine pyrophosphate present in the supernatant since no thiamine pyrophosphate had been added to the incubation mixture. As shown in Table II, the addition of thiamine pyrophosphate from 0.1 to 2.5 mM to the incubation mixture had no effect on thiamine triphosphate-synthesizing activity. At thiamine pyrophosphate concentrations between 2.5 and 5 mM, some slight inhibitor effect was observed. When thiamine [^{32}P]pyrophosphate was used as substrate and the experiment repeated except unlabelled ATP was used instead of the labelled nucleotide, no thiamine [^{32}P]triphosphate was synthesized in any of the subcellular fractions.

When a liver supernatant was dialyzed overnight, only 20—30% of the activity was lost. This finding also supports our suggestion that the endogenous thiamine pyrophosphate might be bound to some species of protein that could not be removed on dialysis.

TABLE I

SUBCELLULAR DISTRIBUTION OF THIAMINE TRIPHOSPHATE-SYNTHESIZING ACTIVITY IN RAT LIVER

Rat liver homogenate and the indicated fractions obtained after differential centrifugations were assayed for thiamine triphosphate-synthesizing activity using [γ-^{32}P] ATP as described in Materials and Methods. Values are the mean of two or three preparations.

Fraction	nmol thiamine triphosphate formed per mg protein	Total activity (nmol thiamine pyrophosphate formed/ fraction)
Homogenate	1.28	2304
Nuclei	0.20	108
Mitochondria	0.17	22
Microsomes	0.08	13
Supernatant	21.2	6487

TABLE II

EFFECT OF ADDED THIAMINE PYROPHOSPHATE ON THIAMINE TRIPHOSPHATE SYNTHESIS IN RAT LIVER SUPERNATANT

A 100 000 × *g* liver supernatant fraction was assayed for thiamine triphosphate-synthesizing activity as described in the text. The incubations were carried out in 50 mM potassium phosphate buffer, pH 6.0, for 30 min at 37°C. The final specific activity of [^{32}P]ATP in the reaction mixture was 450 cpm/nmol. 100% activity was 21.6 nmol of thiamine triphosphate synthesized/mg protein.

Thiamine pyrophosphate added (mM)	Relative activity (%)
0	100
0.1	98
0.5	101
1.0	104
2.5	87
5.0	60

A second approach was employed to investigate the requirement of endogenous thiamine pyrophosphate for thiamine triphosphate synthesis. [^{35}S]Thiamine was injected into a thiamine-deficient rat and the radioactivity in each subcellular fraction was measured. It was found that 80—85% of the total radioactivity was located in the supernatant fraction. Fig. 1 shows the elution profile of the radioactive supernatant fraction when applied to a Sephadex G-75 column (1.2 × 45 cm) in 10 mM potassium phosphate buffer, pH 6.5. Fractions (1.5 ml) were collected and measured for protein & radioactivity: the thiamine content was determined by conversion to its fluorescent thiochrome derivative [15]. Most of the radioactivity came out with the protein peak at

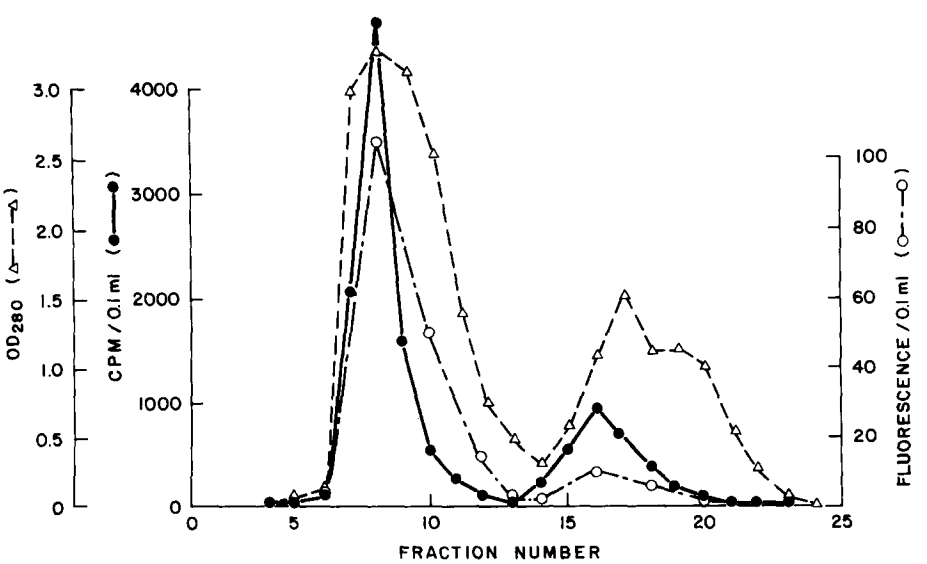

Fig. 1. Elution profile of protein, radioactivity, and thiamine fluorescence of liver supernatant fraction. After injecting labelled thiamine into a rat, the liver supernatant was applied to a Sephadex G-75 column (1.2 × 45 cm), equilibrated with potassium phosphate buffer (10 mM), pH 6.5. Fractions (1.5 ml) were measured for protein (△- - - - -△), radioactivity (●——●), and fluorescence in arbitrary units (○- . - . - .○).

TABLE III

THIAMINE TRIPHOSPHATE SYNTHESIS WITH ^{35}S-LABELLED RAT LIVER SUPERNATANT AND DEPENDENCY ON ATP AND Mg^{2+}

The incubation mixture in total volume of 0.1 ml contained 50 mM potassium phosphate buffer, pH 6.0, 5 mM ATP, 5 mM Mg^{2+} and 1.2 mg labelled protein. The incubations were carried out at 37°C for 30 min and radioactive thiamine triphosphate was measured as indicated in Materials and Methods. Values are means of three experiments.

	[^{35}S] Thiamine triphosphate (cpm/mg protein)
Experimental	3125
—ATP	340
—Mg^{2+}	640
—ATP and —Mg^{2+}	235
+ thiamine pyrophosphate (2.5 mM)	3035

void volume, with a small activity associated with a second protein peak during elution. When the supernatant was dialyzed overnight against the same buffer prior to applying to a column, the second peak of radioactivity and most of the protein was abolished: the same amount of radioactivity still remained attached to the protein peak at the void volume. When this radioactive peak was pooled, deproteinized with trichloroacetic acid and subjected to electrophoresis as described in Materials and Methods, it was found that about 80% of the total radioactivity was in the form of thiamine pyrophosphate. As shown in Table III, when the labelled supernatant was incubated with ATP and Mg^{2+}, radioactive thiamine triphosphate was produced similar to the experiment shown in Table I in which a liver supernatant and [^{32}P] ATP were used.

The concentration dependency of the liver supernatant on thiamine triphosphate synthesis using [^{32}P] ATP and [^{35}S] thiamine, respectively, was then explored. In both cases there was a linear dependency on concentration up to 3.2 mg protein. However, it should be recognized that increasing the super-

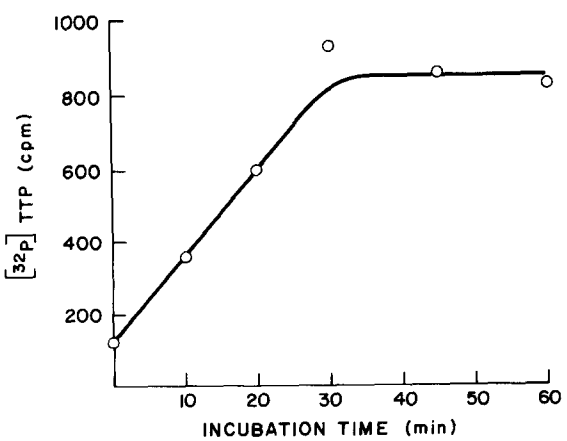

Fig. 2. Time dependency of thiamine [^{32}P] triphosphate synthesis in rat liver supernatant using [^{32}P]-ATP. Assay as described in Materials and Methods using 1.5 mg protein per tube.

natant concentration increases the substrate as well as the enzyme. Thus, we do not know what is rate limiting in the reaction.

Thiamine triphosphate synthesis showed a linear time course up to 30 min (Fig. 2). The effect of a variety of agents on thiamine triphosphate synthesis was investigated. Dithiothreitol, *p*-chloromercuribenzoate, *N*-ethylmaleimide, Ca^{2+}, or cyclic GMP exhibited neither stimulatory nor inhibitory effects.

That the product of the reaction is thiamine triphosphate has been shown not only by the electrophoretic assay but also by paper chromatography. Using a solvent system (isobutyric acid/NH_4OH/NaH_2PO_4/H_2O (198 : 3 : 40 : 59, v/v)) that separates thiamine phosphates and adenine nucleotides, and Whatman No. 3 paper, the R_F value of thiamine [^{32}P] triphosphate was identical to that of the standard and had the same radioactivity content as was determined in the electrophoretic assay.

Discussion

Since repeated attempts to isolate and characterize the thiamine pyrophosphate-ATP phosphoryltransferase from brain particulate fractions were unsuccessful, partially due to contaminating ATPase and thiamine triphosphatase activities in the preparation, we decided to switch to liver where fractionation is simpler. We were fortunate in this choice in that the enzyme activity is in the soluble fraction of the liver cell. In terms of total activity as shown in Table I, the low amount in the homogenate as compared to the supernatant is probably due to the presence of thiamine triphosphatase activity in the homogenate. The localization of activity in the cytosol enabled us to develop a convenient radiometric assay using [γ-^{32}P] ATP.

We had originally planned to use thiamine [^{32}P] pyrophosphate as substrate so that it was something of a surprise for us to discover that free thiamine pyrophosphate was not the substrate. As shown in this study, the actual substrate is a liver supernatant fraction that is either dialyzed or put through a Sephadex G-75 column which contains bound thiamine pyrophosphate. This fraction also contains the phosphoryltransferase activity. Whether this represents a large macromolecular complex with both activities or a separate "substrate • protein" and "enzyme • protein" has not yet been determined. Preliminary attempts to separate these activities using either ammonium sulfate precipitation or DEAE-Sephadex chromatography have resulted in the complete loss of activity.

Although incubating a liver supernatant with ATP and labelled thiamine pyrophosphate does not produce radioactive thiamine triphosphate, we have found that if [^{35}S] thiamine is incubated with the supernatant fraction and ATP, a small amount of labelled thiamine triphosphate is formed. This finding is not surprising since thiamine pyrophosphokinase, the enzyme that catalyzes the synthesis of thiamine pyrophosphate in a one-step pyrophosphorylation of thiamine, is a soluble enzyme, present in this fraction.

Among the agents that were tested for either stimulatory or inhibitory activity was cyclic GMP, since a recent report indicates a stimulatory effect of this cyclic nucleotide on thiamine triphosphate synthesis when $^{32}P_i$ was incubated with a dorsal root preparation [17]. In our system, however, we could find no effect of cyclic GMP. Similarly, the lack of effect of agents that

react with sulfhydryl or disulfide groups suggest that these groups are not involved in either the thiamine pyrophosphate · protein complex or the enzyme activity.

Since we have shown [6] that the "inhibitor" found in body fluids of patients with Leigh's disease only affects the brain phosphoryltransferase and not the liver enzyme, our obvious interest lies in isolating and characterizing the enzyme from brain. Preliminary experiments with a brain particulate fraction indicate that in this tissue the substrate is also not free thiamine pyrophosphate but thiamine pyrophosphate that is bound to this fraction.

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