Hydrolysis and Synthesis of Thiamin Triphosphate in Bacteria

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Summary Thiamin triphosphate (ThTP) in early stationary phase cells of Escherichia coli grown in nutrient broth with 0.1% yeast extract was found to constitute approximately 5–7% of cellular thiamin diphosphate (ThDP) or around 5 nmol/g cell. Nearly the same level of ThTP was obtained in a Bacillus strain. When E. coli was loaded with an excess of ThTP or ThDP, cellular ThTP was found to be controlled in the course of the long term to maintain its ratio to the amount of cellular ThDP. The ThTP vs. ThDP ratio in E. coli cells after short-term ThDP uptake was found to be a function of the cellular growth phase. The ratio in early exponential phase E. coli cells was found to be approximately 4% and it became lower (less than 3%) when cell growth proceeded to the late exponential stage. Two phosphatases specific for ThTP (ThTPase) among thiamin phosphates were detected in E. coli. One required Mg²⁺ and was found mainly in the soluble fraction, while the other was Mg²⁺-independent and originated from the membrane. The two ThTPases were similar to their rat tissue counterparts.

Key Words thiamin triphosphate, thiamin triphosphatase, thiamin diphosphate kinase, thiamin pyrophosphate, thiamin, E. coli

Recently, data on thiamin triphosphate (ThTP) in the tissues of higher organisms as determined by high performance liquid chromatography (HPLC) have been accumulating (1, 2). Those recent reports have reconfirmed earlier discoveries of ThTP in rat tissues (3, 4), in yeast (5) and in plant cells (6). In spite of the detections of a small but consistent amount of ThTP, including an exceptionally high percentage of ThTP among the thiamin phosphates in an electric organ of Torpedo marmorata (7) and pig muscle (8), its biological significance has yet to be established. Recent studies on ThTP have mainly focussed on its role in the nervous system.
system of higher organisms (e.g., 9), and no report on ThTP metabolism in bacteria has been published.

So far there has been only one report on ThTP in bacterial cells: an earlier study on Mycobacterium lacticola (10) using ion exchange chromatography. Our HPLC study gave very similar results. Moreover, cellular ThTP was found to be in dynamic equilibrium between its synthesis and breakdown. ThTP uptake experiments proved its rapid hydrolysis to thiamin diphosphate (ThDP). When E. coli accumulated ThDP, conversely, a parallel increase in cellular ThTP occurred. ThTP-hydrolyzing enzymes have been reported in soluble and membrane fractions of rat brain (9, 11–14). We report here on two ThTPases of E. coli.

MATERIALS AND METHODS

**Strains.** Escherichia coli W 1485, and a thiamin-requiring mutant of E. coli W (70-23) together with its thiamin-kinase mutant (70-23-102) and ThMP-kinase mutant (70-23-107) (15) were used. A thiaminase-producing bacillus, Bacillus aneurinolyticus KA30, is an original isolate in the Department of Microbiology, Yamaguchi Univ. School of Medicine (16).

**Growth conditions.** Davis's minimal medium (17) was used in double strength supplemented with 5 × 10^{-9}–10^{-8} M thiamin and other additives when specified, for experiments and large-scale cultures. Bacillus aneurinolyticus KA30 was grown in a 1:1 mixture of Schaeffer's sporulation medium (18) and nutrient broth (Nissui) supplemented with 0.1% yeast extract (Difco). The amount of cells was determined by spectrophotometric measurement and expressed in gram dry weight.

**High pressure liquid chromatography.** ThTP was detected by reverse phase HPLC using a LiChrosorb RP18 (E. Merck Co.) 4 mm × 250 mm column. The developing solvent was 2.5% C2H5OH in 25 mM potassium phosphate buffer, pH 7.6. The detection was accomplished in a 90-μl flow cell with an excitation wave of 375 nm and fluorescence emission of 430 nm. The minimum amount detected was approximately 0.05 pmol. For sample preparation, cells were first washed with cold 0.01 M Tris-HCl, pH 7.4, containing 0.03 M KCl (W buffer), and cells from 100 ml of culture were extracted in 1.0 ml of 0.1 M sodium acetate buffer, pH 4.0 (E buffer), by heating at 100°C for 5 min and then at 80°C for 10 min. After cooling, 10% cold trichloroacetic acid (TCA) was added to the concentration, centrifuged at 5,000 × g for 5 min in the cold, and the supernatant was extracted three times with ethyl ether. The water layer was neutralized (1 N NaOH) and concentrated to ca. 60 μl by a centrifuging freeze dryer. The exact volume was measured, and an aliquot (10 μl) was oxidized with 10 μl of 0.2% K3Fe(CN)6 and 35 μl of 30% NaOH. After 30 min, ca. 35 μl of 7.5 N HCl (to pH ca. 8.4) was added. This original sample was adjusted to a volume of 300 μl with 5 mM sodium acetate buffer, pH 4.0. The HPLC sample analyzed was 0.5–5 μl. BrCN oxidation was also used in a similar manner.

For the sample of Bacillus aneurinolyticus containing spores, 100 ml of H2O-washed cells were suspended in 1.0 ml of E buffer. Five ml of boiling n-propanol was
added, heated for 5 min at 97°C (19), and centrifuged at 3,000 × g for 5 min at 4°C. The supernatant was TCA precipitated and oxidized as above.

**ThTP uptake by E. coli.** Late exponential phase E. coli cells washed with cold W buffer were incubated with 10⁻⁵ M ThTP, 0.4% glucose, and 0.1 M sodium phosphate buffer of pH 7.2 at a cell concentration of 3.3 mg/ml at 37°C for 15 min aerobically. At the end of the incubation, it was poured on a filter (0.45 μm) and washed with ice-cold 0.1 M phosphate buffer, pH 7.2. Thiamin phosphates were extracted in 7.0 ml of E buffer and concentrated to 3.5 ml. It was divided into 2 equal volumes for thiochromes and non-thiamin fluorescence control, and analyzed using the gel filtration method (20).

**ThDP uptake experiment.** Early (A₄₂₀ = 0.1) or late (A₄₂₀ = 1.0) exponential phase E. coli W70-23 or W70-23-107 cells were washed as described above and incubated with 10⁻⁴ M ThDP, 0.4% glucose, and 0.1 M sodium phosphate buffer, pH 7.2 (1.3 mg cell/ml), at 37°C. At specified times, the reaction mixture was chilled to 0°C and centrifuged for 5 min. The pellet was washed with 4 ml of cold 0.033 M Tris-HCl, pH 7.4, and extracted with 1 ml of E buffer to prepare the sample for HPLC.

**Preparation of ThTPases.** E. coli W70-23 cells were grown as above in one liter of medium, pelleted, washed with W buffer at 4°C, and shocked osmotically by the method of Neu and Heppel (21). The shocked cells were dispersed in 40 ml of 10 mM Tris-HCl, pH 7.4, containing 0.5 mM 2-mercaptoethanol (A buffer) and disrupted in milder conditions by sonication. The undisrupted cells were spun down and washed with 30 ml of A buffer. From the supernatant and the washing combined, the membrane fraction was collected by 150,000 × g centrifugation at 4°C for 120 min and washed with A buffer.

**Mg²⁺-Dependent ThTPase I preparation.** The membrane fraction obtained above was suspended in 6 ml of A buffer. Triton X100 (1.5% by volume) was added and the mixture was shaken gently at 30°C for 20 min. Mg²⁺-ThTPase was obtained in the 150,000 × g supernatant at 4°C and dialyzed immediately against A buffer.

**Mg²⁺-Independent ThTPase II preparation.** To the membrane fraction in 6 ml of A buffer, 5 M NaClO₄ (in 0.1 M Tris-HCl, pH 7.4, 0.5 mM 2-mercaptoethanol and 1 mM EDTA) was added to give 0.5 M NaClO₄ and shaken gently for 20 min at 30°C. The solubilized ThTPase in the supernatant after 150,000 × g centrifugation was dialyzed immediately against half-strength A buffer at 4°C.

**Assay of ThTPases.** For ThTPase I, the 50-μl mixture contained enzyme, 50 mM Na cacodylate buffer (pH 7.2), 4 mM MgCl₂, 2 mM ThTP, and 0.1 mM 2-mercaptoethanol. It was incubated at 42°C for 60 min. The 50-μl mixture for ThTPase II contained enzyme, 100 mM Tris-maleate buffer (pH 6.5), 40 mM KCl, 0.5 mM ThTP, and 0.1 mM 2-mercaptoethanol and was incubated at 37°C for 1 h. After the reaction, 200 μl of 20% cold TCA was added and spun down in the cold. The Pi in the supernatant was determined by the modified Fiske-Subbarow’s method of Baginski et al. (22) or by the method of Concustell et al. (23). The reaction proceeded linearly with time for at least 2 h in ThTPase I and for approximately 3 h in ThTPase II.
Assay of other phosphatases. For Mg\(^{2+}\)-ATPase, 0.5 ml of the reaction mixture contained 100 mM Tris-HCl (pH 8.5), 1 mM MgCl\(_2\), 2 mM ATP, 0.1 mM 2-mercaptoethanol, and sample, and was incubated at 37°C for 60 min. 5'-Nucleotidase (24), glucose-6-phosphatase (21), and alkaline p-nitrophenylphosphatase (25) were assayed following previously reported methods. In the assay of acid p-nitrophenylphosphatase, the substrate was incubated in the mixture for 5'-nucleotidase. The Pi liberated was determined as in ThTPases. Protein was determined by the method of Biuret (26) or Lowry (27). ThTP was the generous gift of Dr. M. Yamazaki of Sankyo Co., Tokyo.

RESULTS

Amount of ThTP in E. coli

Approximately 3–7 nmol/g of ThTP was always detected in E. coli grown in nutrient broth supplemented with 0.1% yeast extract. The HPLC peak of ThTP was identified (i) by the use of authentic ThTP detected at the same retention time; (ii) by recovering the eluate of the peak HPLC fraction and taking the excitation and emission wavelength spectra (375 nm and 430 nm, respectively); and (iii) by the disappearance of the ThTP peak when alkali was added before BrCN or K\(_3\)Fe(CN)\(_6\). The ThDP (Nakarai Chemicals Co.) added to the media was analyzed for ThTP and it was found to be less than 0.14% (mol/mol) of ThDP.

When the same cells were grown in a synthetic minimal medium, 10\(^{-7}\)–5 \times 10\(^{-7}\) M thiamin or ThDP had to be added to the medium to obtain the same level of cellular ThTP. When the concentration of thiamin in the medium was varied from 10\(^{-8}\) to 10\(^{-3}\) M, ThTP in late exponential phase cells was found to increase along with the cellular ThDP (Fig. 1a). The relative amount of ThTP to ThDP was in the approximate range of 5–7% (mol/mol; cellular ThDP was in the concentration range below 2 \(\mu\)mol/g). The relative amount of ThMP compared to ThDP varied between 18% and 40% (mol/mol) (Fig. 1a). In this report, ThTP is mostly presented as a percentage value against that of ThDP. A rough estimate of the percentage of thiamin and its phosphates in the total thiamin in E. coli W70-23 grown in a minimal medium supplemented with 10\(^{-5}\) M thiamin was 3.8% ThTP, 65.4% ThDP, 23.6% ThMP, and 7.2% thiamin.

ThTP content after in vitro uptake of ThDP or ThTP

The relative amount of ThTP to ThDP mentioned above is the value in equilibrium. To determine the adjusting activity of the ThTP-ThDP ratio in E. coli, ThDP or ThTP was loaded by an in vitro uptake reaction. As shown in Fig. 1b, ThDP accumulation over a short period of time did not yield the equilibrium level of ThTP. Instead, the accumulated ThTP constituted about 4% of cellular ThDP. In a paired experiment, 0.6 \(\mu\)mol/g cell of ThDP was attained by growing the same E. coli cells for 2.5 h in the minimal medium supplemented with 5 \times 10\(^{-5}\) M thiamin instead of using the E. coli in the uptake reaction. The measured ThTP in such cells
Fig. 1. a: Relative amounts of cellular ThDP and ThTP in E. coli. E. coli W70-23 was grown in double-strength minimal medium with various concentrations of ThDP. ThTP values are multiplied ten times for clearer plotting. Data for nutrient broth (0.1% yeast extract) cultures of E. coli W70-23, K12, and W fitted well on the drawn curve if it was plotted at the corresponding ThDP values. b: ThDP loading on early exponential phase E. coli cells. A preculture of E. coli W70-23 in double-strength minimal medium supplemented with 10^{-8} M thiamin was transferred into the same medium at 1:60 dilution and cultured for approximately 2.5 h. When A_{420} = 0.10, the cells were collected, washed, and used in the uptake experiment of 10^{-4} M ThDP. Values for ThTP are multiplied ten times for clearer plotting.

Table 1. Fate of thiamin triphosphate in Escherichia coli after 15-min uptake reaction.

<table>
<thead>
<tr>
<th>Total uptake (nmol/g · h)</th>
<th>Relative amount (mol/mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ThTP^b</td>
</tr>
<tr>
<td>Complete reaction^a</td>
<td>676 ± 41</td>
</tr>
<tr>
<td>Complete – glucose</td>
<td>368</td>
</tr>
<tr>
<td>Complete + 0.1 M KF</td>
<td>174</td>
</tr>
</tbody>
</table>

^aAverage of three independent experiments carried out as in METHODS using E. coli W70-23. An experiment with E. coli W1485 gave similar results. ^bE. coli cells were grown in the minimal medium with 5 × 10^{-9} M thiamin and the endogenous ThTP was less than 0.2 nmol/g.

was 5.2% vs. ThDP, within the range of equilibrium described above, indicating its attainment over a longer period of time.

When ThTP was added extracellularly, E. coli mainly accumulated ThDP (Table 1). The ratio of ThTP to ThDP (44-47%) was far out of balance from the point of equilibrium observed above. This result indicates that cellular ThTP passed
through the envelope of *E. coli* without dephosphorylation. ThDP, which can be produced extracellularly, has been shown to be incorporated into this strain without dephosphorylation (20). In the uptake of ThDP, the percentage of cellular ThTP was shown above (Fig. 1b) to be much lower than in Table 1. It is already accepted that free thiamin accumulates in *E. coli* cells not in the form of ThTP but mainly as ThDP. On the other hand, we could not estimate what percentage of ThDP was transported and accumulated in this form, or produced through ThTP dephosphorylation or through free thiamin uptake, but we deduce that a certain portion of ThDP was the product of intracellular hydrolysis of ThTP because of the high ThTP content in the cells.

**Growth phase and ThTP level**

In the ThDP loading test (Fig. 1b), *E. coli* cells in the early exponential phase
were used. When the experiment was carried out using late log phase or early stationary phase cells, ThTP was always below 3% of ThDP. Hence, the effect of growth phase on the rate of ThTP synthesis from ThDP was studied. E. coli cells in various growth phases (Fig. 2a) were loaded with ThDP through a 90-min uptake reaction and thiamin phosphates were determined. Higher activity of ThTP synthesis in the early exponential phase cells was confirmed in the ratio of ThTP/ThDP (Fig. 2a). This result was partly attributable to the fact that the rate and maximum amount of ThDP uptake became higher when the growth approached the late exponential phase, while the conversion rate into ThTP remained relatively constant.

The results of ThDP uptake using late exponential phase cells (Fig. 2b) confirmed the above notion. In this experiment, a higher level of ThTP had accumulated than that shown in Fig. 1b (approximately two fold), but the accumulation rate of ThDP exceeded by far the conversion rate. Consequently, the percentage of ThTP remained at approximately 2.6% (mol/mol) of ThDP within the short reaction time. The E. coli cells used in this experiment could not phosphorylate ThMP to ThDP. Hence cellular ThDP was either the intracellular dephosphorylation product of ThTP or that incorporated from outside. In this mutant, extracellular ThDP phosphatase activity is enhanced compared to that in E. coli W70-23. The accumulation of ThMP has been shown to be the result of extracellular dephosphorylation of ThDP into free thiamin, its subsequent accumulation, and finally its intracellular monophosphorylation (20). In strain W70-23, ThDP is incorporated as it is (20).

**ThTP in Bacillus aneurinolyticus**

Typical results of ThTP determination are shown in Table 2. In both early exponential phase and stationary phase cells, the relative amount of ThTP was 5.0–6.4% (mol/mol) of ThDP.

**ThTPases of Escherichia coli**

From the data in Table 1, a monophosphatase was inferred to be active on ThTP, and we tried to detect it. When E. coli was shocked osmotically, a large part

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>ThTP</th>
<th>ThDP (nmol/g)</th>
<th>ThMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-h culture</td>
<td>2.73 ± 0.55</td>
<td>54.6 ± 0.84</td>
<td>6.03 ± 0.90</td>
</tr>
<tr>
<td>61-h culture</td>
<td>2.83 ± 1.50</td>
<td>43.7 ± 0.84</td>
<td>6.53 ± 0.75</td>
</tr>
</tbody>
</table>

*Early exponential phase cell. Stationary phase cell. Data are averages of three independent experiments.*
Table 3. Distribution of activities among subcellular fractions of *E. coli*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ThTPase I (Sp. act.)</th>
<th>ThTPase II (Sp. act.)</th>
<th>Mg$^{2+}$-ATPase</th>
<th>AMPase$^a$</th>
<th>G6Pase$^b$</th>
<th>pNPPase$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicated cells</td>
<td>24,500 (148)</td>
<td>9,640 (41)</td>
<td>343,000</td>
<td>10,000</td>
<td>4,420</td>
<td>19,600</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>7,440 (269)</td>
<td>2,190 (79)</td>
<td>50,200</td>
<td>1,560</td>
<td>660</td>
<td>1,180</td>
</tr>
<tr>
<td>Soluble supernatant</td>
<td>13,000 (77)</td>
<td>612 (3.6)</td>
<td>915,000</td>
<td>—</td>
<td>6,680</td>
<td>48,300</td>
</tr>
<tr>
<td>Solubilized from membrane</td>
<td>2,680 (543)</td>
<td>1,000 (544)</td>
<td>532</td>
<td>1,190</td>
<td>1,300</td>
<td>3,000</td>
</tr>
<tr>
<td>Shock fluid</td>
<td>33,900$^d$</td>
<td>14,300$^d$</td>
<td>40,000</td>
<td>1,830,000</td>
<td>90,400</td>
<td>83,200</td>
</tr>
</tbody>
</table>

$^a$ 5'-Nucleotidase. $^b$ Glucose-6-phosphatase. $^c$ Acid p-nitrophenyl phosphatase (pH 5.8) with Co$^{2+}$ and Ca$^{2+}$ (total activity for pH 9.0 pNPPase (25) was less than 1/5). $^d$ Nonspecific phosphatases gave part of this activity. Specific activity (Sp. act.) is expressed in μmol/g·h. ThTPase I and II were both assayed at 42°C for 1 h.

of various periplasmic phosphatases was in the shock fluid whereas the major fraction of membrane Mg$^{2+}$-ATPase [EC 3.6.1.3] was recovered with the shocked (sonicated) cells (Table 3). ThTPase showed an intermediate distribution between them. Since an overwhelming excess of nonspecific phosphatases was found in the shock fluid, it is likely that at least part of the apparent ThTPase activity in the shock fluid was the result of a side reaction of those phosphatases.

When the cells were disrupted and the soluble fraction was separated from the membranous proteins by 150,000 × g centrifugation for 2 h, it became clear that the ThTPase in the supernatant behaved differently from the enzyme in the membrane fraction in terms of dependency upon Mg$^{2+}$ (Fig. 3, a and b) and in the effect of substrate concentration. The supernatant enzyme (Figs. 3a and 4a) exhibited a substrate inhibition at higher ThTP concentration (Fig. 4a), but the membrane enzyme (Fig. 4b) did not. The $K_m$ for membrane enzyme was smaller (0.07 mM) than that for the supernatant enzyme (0.9 mM). The effect of pH was also different (Fig. 5a). This result of optimum pH showed that the Mg$^{2+}$-dependent supernatant enzyme (ThTPase I) was different from Mg$^{2+}$-ATPase (which showed an activity peak at approximately pH 9; data not shown). As in Table 3, the supernatant was relatively concentrated with Mg$^{2+}$-dependent ThTPase I, whereas the membrane fraction contained the major activity of Mg$^{2+}$-independent enzyme (ThTPase II). This ThTPase II was solubilized from the particulate fraction. Mg$^{2+}$-ThTPase I in the soluble fraction was accompanied by a large excess (approximately 70 times) of Mg$^{2+}$-ATPase [EC 3.6.1.3] (Table 3) in our sonication conditions. To avoid this, an enzyme which showed Mg$^{2+}$ dependency and substrate inhibition like Mg$^{2+}$-ThTPase I was solubilized from the membrane with less contamination. Solubilized enzyme had practically the same characteristics as soluble Mg$^{2+}$-ThTPase I and it

Fig. 3. Effect of Mg\(^{2+}\) on crude ThTPase I and membraneous ThTPase II. Late exponential phase E. coli W70-23 cells grown in double-strength minimal medium supplemented with 5 × 10\(^{-9}\) M thiamin were used for the preparation of enzymes. Enzymes solubilized as in METHODS were used in the amounts of 170 μg and 32 μg protein for ThTPase I and ThTPase II, respectively. The reaction mixture contained the same components as in METHODS except for various concentrations of MgCl\(_2\) and KCl and 1 mM ThTP instead of 0.5 mM in ThTPase II. a: Mg\(^{2+}\)-dependent crude ThTPase I; b: Mg\(^{2+}\)-independent membrane-bound ThTPase II.

Fig. 4. Effect of substrate (ThTP) on ThTPase I and ThTPase II. Enzyme preparations were obtained as described in the legend to Fig. 3. In the ThTPase I experiment, 100% corresponded to 0.50 mmol P\(_i\)/g·h. Activity at 2 mM ThTP was arbitrarily designated as 100%. For ThTPase II, the reaction mixture contained various concentrations of ThTP. a: Mg\(^{2+}\)-dependent soluble ThTPase I; b: Mg\(^{2+}\)-independent membrane-bound ThTPase II (K\(_m\) = 7.1 × 10\(^{-5}\) M).

was different from Mg\(^{2+}\)-ATPase electrophoretically in polyacrylamide gel (data not shown). The latter enzyme expressed substantially no activity on ThTP. The efficiency of solubilization of the two ThTPases was compared at 30°C for 20 min.
Fig. 5. Effect of pH and temperature on crude ThTPase I and membraneous ThTPase.

a: Effect of pH. ThTPase I was prepared as in Fig. 3 but the enzyme was solubilized from the membrane by 0.4 M NaSCN instead of 15% Triton X100 and dialyzed against buffer A. ThTPase II was prepared as described in Fig. 3. The buffers used were Tris-maleate for pH less than 7.5 and Tris-HCl for pH above 7.6. The reaction mixture contained the components described in MATERIALS AND METHODS except for buffer components of altered pH.
b: Effect of temperature. ThTPase I and II were prepared as in Fig. 3.

Table 4. Effect of metal ion on ThTPase.

<table>
<thead>
<tr>
<th></th>
<th>Crude ThTPase I (with 4 mM Mg²⁺)</th>
<th>Membraneous ThTPase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100ᵃ</td>
<td>100ᵇ</td>
</tr>
<tr>
<td>5 mM MgCl₂</td>
<td>NT</td>
<td>66</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>46</td>
<td>81</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>72</td>
<td>29</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>29</td>
<td>77</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>28</td>
<td>78</td>
</tr>
<tr>
<td>PbCl₂</td>
<td>NT</td>
<td>40</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>44</td>
<td>73</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>11</td>
<td>51</td>
</tr>
<tr>
<td>40 mM LiCl</td>
<td>39</td>
<td>80</td>
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<tr>
<td>NaCl</td>
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<td>KCl</td>
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<td>124</td>
</tr>
<tr>
<td>RbCl</td>
<td>29</td>
<td>91</td>
</tr>
<tr>
<td>CsCl</td>
<td>NT</td>
<td>62</td>
</tr>
</tbody>
</table>

ᵃ 100% corresponded to 543 μmol/g·h. ᵇ 100% corresponded to 236 μmol/g·h. NT, not tested. Standard assay conditions are described in MATERIALS AND METHODS.

using several reagents. With 0.5 M NaClO₄, 46% ThTPase II and less than 10% of ThTPase I were extracted whereas 1.5% Triton X100 solubilized 36% ThTPase I and 16% ThTPase II of very low specific activity. 0.5 M sodium deoxycholate was

Table 5. Substrate specificity of ThTPase I.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Activity</th>
<th>Substrate</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThTP</td>
<td>100%</td>
<td>None</td>
<td>100%</td>
</tr>
<tr>
<td>ThDP</td>
<td>2.6</td>
<td>2 mM ATP</td>
<td>116</td>
</tr>
<tr>
<td>ThMP</td>
<td>0</td>
<td>0.2 mM ADP</td>
<td>57.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 mM ADP</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM pyrithiamin</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 mM pyrithiamin</td>
<td>7.9</td>
</tr>
</tbody>
</table>

*100% corresponded to 312 μmol/g·h. *b 100% corresponded to 498 μmol/g·h. Standard assay conditions are described in MATERIALS AND METHODS.

not as effective as the two reagents. The solubilized enzyme was used throughout this study in place of the supernatant enzyme.

**Other characteristics of ThTPases**

The effect of metal ions and cations was studied using solubilized ThTPases (Table 4). In ThTPase I, inhibition was observed in metal ions except for Mg²⁺ and K⁺. In ThTPase II, activation was detected in K⁺, and Cu²⁺, Pb²⁺, and Zn²⁺ inhibited the activity. The extent of activation of ThTPase II (Fig. 3b) by K⁺ varied between cell preparations. The results in Table 4 indicated that they were different from p-nitrophenyl phosphatase (in the effect of Mg²⁺, Co²⁺, Cu²⁺), glucose-6-phosphatase (in the effect of Mn²⁺, etc.) or 5’-nucleotidase (in the effect of Co²⁺ and Ca²⁺ (25)).

In ThTPase I, the effect of the ratio of Mg²⁺ to ThTP was studied with 2 mM ThTP and varying concentrations of MgCl₂. The optimum ratio was found to be 2, and the activity decreased linearly until it was almost zero at the ratio of 20. Substrate specificities and the effects of some thiamin analogs are shown in Table 5. ThTPase I showed sensitivity to (neo)pyrithiamin (Sigma Chemical Co.) and ADP.

**DISCUSSION**

The established role of thiamin diphosphate as a coenzyme indicates that ThDP is involved in those basic and well-conserved pathways developed very early phylogenetically. From this point of view, the physiological role of ThTP, if any, may be expected in prokaryotic cells in addition to its postulated importance in the nervous system of higher organisms.

The level of ThTP in bacterial cells was too low to detect with the HPLC system used when the cells were grown in a minimal medium. The existence of bacterial ThTP was confirmed using culture media that contained routinely adopted amounts of yeast extract. In the case of minimal medium-grown *E. coli* cells, we had to supplement thiamin at about 20 times the concentration of the minimal
requirement. Whether a definite but minute amount of ThTP is present in the minimal medium-grown *E. coli* or whether such cells are totally devoid of ThTP remains to be established. If the latter is the case, the presence of ThTP is dispensable for the growth of *E. coli*. In such a case, it is still possible that ThTP is a regulatory factor in conditions of relative thiamin abundance. The possibility must also be considered that ThTP is a side product of high thiamin availability.

Actively growing cells of *E. coli* were found to be in a state of equilibrium in ThTP-ThDP balance (Fig. 1a). In experiments on both ThTP and ThDP loading, it was observed that a deviation from the equilibrium between ThTP and ThDP was being corrected during the course of time (Fig. 1b and Table 1; see text). In the case of ThDP loading, the rate of ThDP uptake slows down gradually during the course of incubation (attaining equilibrium between the uptake and exit (28)), while the conversion rate of ThDP into ThTP seemed to continue at nearly the same rate (Figs. 1b and 2b). Thus the relative amount of ThTP increases gradually to approach the level of equilibrium.

In ThDP uptake experiments, the maximum possible concentration of contaminating ThTP in the ThDP reagent (less than 0.14%) is high enough when the ThDP is at the level of $10^{-4}$ M. Thus it is conceivable that cellular ThTP is the result of uptake of extracellular molecules. In this case, however, it has to be assumed that *E. coli* has a specific mechanism for the selective concentration of ThTP (from less than 0.14% ThTP vs. ThDP to approximately 4%) among thiamin phosphates amounting to more than 30 times its concentration. This seems less likely since it is known that ThMP or thiamin competes against ThDP for a single uptake system in *E. coli* and shares a common pathway (29). In any event, the equilibrium concentration of 5–7% ThTP vs. ThDP is not the result of simple uptake, and it can only be attained through a longer incubation, most likely with the phosphorylation of cellular ThDP.

*Mycobacterium lacticola* contained 7.1–11.2% of ThTP against ThDP (10). This value is slightly higher than in *E. coli* or in *B. aneurinolyticus*, but the experimental method was different (assay with Dowex 50 column chromatography for *Mycobacteria*). Interestingly, ThTP in *Mycobacterium* decreased in the course of growth stage from 1.3 μg/mg protein N at 7 days culture to 0.5 μg/mg protein N at 13 days culture (10). In *E. coli* cells, ThDP-ThTP conversion activity in various growth phases was relatively constant throughout the growth phase in a wider range of activation of ThDP uptake. In Fig. 2b (late exponential cells), the rate of ThDP uptake had increased approximately 3 times compared to Fig. 1b (early exponential cells), and the rate of ThDP-ThTP conversion was approximately two fold. From the fact that the rate of ThTP increase was relatively constant throughout the reaction periods (Figs. 1b and 2b), during which time ThDP continued to increase, the converting enzyme seemed to be saturated with cellular ThDP. Thus, it was deduced that the total amount of converting enzyme had increased in the late exponential phase. However, the extent of increase was not more than the increase of ThDP uptake activity (Fig. 2, a and b). This fact showed that cellular ThTP was
not the product of a conversion reaction in which the amount of ThTP was determined solely by the concentration of ThDP.

To maintain equilibrium between ThTP and ThDP, ThTP hydrolyzing activity must also play a part. As one of the candidates for adjusting mechanisms at the enzyme level, ThTPase I was found to be highly specific for ThTP among thiamin phosphates (Table 5). This fact may explain the apparent lack of correlation between the amounts of ThMP and ThDP. ThTPase II should also be considered for the possibility of participation in the metabolism of cellular ThTP (30). Two ThTPase activities, first reported in some detail in E. coli, showed close similarities with rat enzymes (9, 11-14) (Table 6). Between the soluble enzymes, major differences were optimal pH and sensitivity to inhibition by pyrithiamin. Among membrane-bound enzymes, rat enzyme was shown to be more sensitive to ATP inhibition and to require divalent ion and a higher concentration of ThTP. In spite of those differences, both eukaryotic and prokaryotic cells contain very similar ThTPases. These results may suggest some common function(s) of cellular ThTP in both types of cells.

REFERENCES


