Pathway of Thiamine Pyrophosphate Synthesis in *Micrococcus denitrificans*

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The pathway of thiamine pyrophosphate (TPP) biosynthesis, which is formed either from exogenously added thiamine or from the pyrimidine and thiazole moieties of thiamine, in *Micrococcus denitrificans* was investigated. The following indirect evidence shows that thiamine pyrophosphokinase (EC 2.7.6.2) catalyzes the synthesis of TPP from thiamine: (i) [35S]thiamine incubated with cells of this microorganism was detected in the form of [35S]TPP or [35S]thiamine; (ii) thiamine gave a much faster rate of TPP synthesis than thiamine monophosphate (TMP) when determined with the extracts; and (iii) a partially purified preparation of the extracts can use thiamine, but not TMP, as the substrate. The activities of the four enzymes involved in TMP synthesis from pyrimidine and thiazole moieties of thiamine were detected in the extracts of *M. denitrificans*. The extracts contained a high activity of the phosphatase, probably specific for TMP. After *M. denitrificans* cells were grown on a minimal medium containing 3 mM adenosine, which causes derepression of de novo thiamine biosynthesis in *Escherichia coli*, the activities of the four enzymes involved with TMP synthesis, the TMP phosphatase, and the thiamine pyrophosphokinase were enhanced two- to threefold. These results indicate that TPP is synthesized directly from thiamine without forming TMP as an intermediate and that de novo synthesis of TPP from the pyrimidine and thiazole moieties involves the formation of TMP, followed by hydrolysis to thiamine, which is then converted to TPP directly. Thus, the pathway of TPP synthesis from TMP synthesized de novo in *M. denitrificans* is different from that found in *E. coli*, in which TMP synthesized de novo is converted directly to TPP without producing thiamine.

It has been well documented that thiamine pyrophosphate (TPP) acts as the coenzyme for pyruvate dehydrogenase (EC 1.2.4.1), oxoglutarate dehydrogenase (EC 1.2.4.2), pyruvate decarboxylase (EC 4.1.1.1), transketolase (EC 2.2.1.1), and several other enzymes. The pathway of TPP biosynthesis in yeast (2-4, 14, 15, 17, 26-28, 31) and *Escherichia coli* (10, 12, 25), has been established and is shown in Fig. 1. The four enzymes involved in the biosynthetic process of thiamine monophosphate (TMP) from pyrimidine and thiazole moieties of thiamine are the same in both organisms, whereas the reactions involved in TPP synthesis from TMP synthesized de novo are different in these two organisms. In yeast, TPP is synthesized from TMP via an intermediate production of free thiamine (2-4); that is, TMP is first dephosphorylated to thiamine before it is converted to TPP. Exogenously added thiamine is, therefore, synthesized to TPP by a one-step reaction catalyzed by thiamine pyrophosphokinase (EC 2.7.6.2) (13, 30, 31). In *E. coli*, TMP is directly phosphorylated to TPP without producing free thiamine and, therefore, thiamine added exogenously is transformed to TPP by two successive steps catalyzed by thiamine monophosphokinase (8, 20, 21) and TMP kinase (20, 21, 23, 24), respectively.

The enzymatic differences in the synthesis of TPP between yeast and *E. coli* prompted us to study the reason why TPP synthesis from thiamine proceeds by a one-step pyrophosphorylation reaction in yeast and, on the other hand, by a two-step phosphorylation reactions in *E. coli*. During the survey of different microorganisms, it was also found that *Micrococcus denitrificans* does contain the thiamine pyrophosphokinase activity.

The present paper describes evidence for the existence of thiamine pyrophosphokinase in *M. denitrificans* as well as the pathway of de novo synthesis of TPP from the pyrimidine and thiazole moieties of thiamine.

**MATERIALS AND METHODS**

Organisms. *M. denitrificans* strain 12442 and *E. coli* K-12 strain 3301 were obtained from the Insti-
PATHWAY OF TPP SYNTHESIS IN M. DENITRIFICANS

Fig. 1. Pathway of TPP synthesis in yeast and M. denitrificans (solid line) and E. coli (dotted line). (I) Hydroxymethylpyrimidine kinase; (II) hydroxymethylpyrimidine phosphate kinase; (III) hydroxethylthiazole kinase; (IV) thiaminephosphate pyrophosphorylase; (V) a phosphatase (nonspecific in yeast; probably specific in M. denitrificans); (VI) thiamine pyrophosphokinase (yeast and M. denitrificans); (VII) thiamine monophosphokinase (E. coli); (VIII) TMP kinase (E. coli). HMP, Hydroxymethylpyrimidine or 2-methyl-4-amino-5-hydroxymethylpyrimidine (thiamine pyrimidine); HMP-P, HMP monophosphate; HMP-PP, HMP pyrophosphate; Th, hydroxethylthiazole or 4-methyl-5-hydroxyethylthiazole (thiamine thiazole); Th-P, Th monophosphate.

1. Pathway of TPP synthesis in yeast and M. denitrificans (solid line) and E. coli (dotted line). (I) Hydroxymethylpyrimidine kinase; (II) hydroxymethylpyrimidine phosphate kinase; (III) hydroxethylthiazole kinase; (IV) thiaminephosphate pyrophosphorylase; (V) a phosphatase (nonspecific in yeast; probably specific in M. denitrificans); (VI) thiamine pyrophosphokinase (yeast and M. denitrificans); (VII) thiamine monophosphokinase (E. coli); (VIII) TMP kinase (E. coli). HMP, Hydroxymethylpyrimidine or 2-methyl-4-amino-5-hydroxymethylpyrimidine (thiamine pyrimidine); HMP-P, HMP monophosphate; HMP-PP, HMP pyrophosphate; Th, hydroxethylthiazole or 4-methyl-5-hydroxyethylthiazole (thiamine thiazole); Th-P, Th monophosphate.

The kinetic activity was expressed as nanomoles of TPP formed per milligram of protein per hour.

Assay of the activities of TMP-synthesizing enzymes. Activities of four enzymes involved in TMP synthesis from hydroxymethylpyrimidine and hydroxethylthiazole (Fig. 1) were determined by procedures described previously (9, 10).

Assay of the activity of TMP-phosphohydrolysing enzyme. The reaction mixture was composed of 0.1 μmol of TMP, 250 μmol of Tris-hydrochloride (pH 7.5), and crude extract (2 mg of protein) in a total volume of 5 ml. The reaction mixture was incubated for 20 min at 37°C, and the amount of thiamine liberated was determined by the thiochrome fluorescence method (7). The reaction rate determined under these conditions was linear up to 5 mg of protein and also up to 60 min of incubation at 37°C.

Analysis of intracellular forms of [35S]thiamine transported. The M. denitrificans cell suspension prepared as described above was incubated with 1 μM [35S]thiamine for 2 and 10 min at 37°C. The fate of [35S]thiamine taken up by M. denitrificans cells was analyzed by the paper chromatographic method. The procedure employed was identical to that used to study the fate of [35S]thiamine or [35S]hydroxethylthiazole transported into E. coli cells (11, 32).

RESULTS

Chromatographic analysis of [35S]thiamine taken up by cells of M. denitrificans. Resting cells of M. denitrificans were incubated with [35S]thiamine in the presence of glucose, and then the fate of [35S]thiamine after entry into the cell was determined by paper chromatographic analysis. The two large radioactivity peaks corresponding to Rf values 0.13 and 0.61 were identified as [35S]TPP and [35S]thiamine,

solution of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.5) and 1 mM dithiothreitol and then resuspended in the same buffer system, followed by sonication treatment for 5 min at 4°C.

After centrifugation for 20 min at 15,000 × g, the supernatant fluids were used as crude extracts for the assay of enzyme activities.

Protein concentration of the extracts was determined by the method of Lowry et al. (18), with bovine serum albumin as a standard.

Assay of the enzyme activity to catalyze the synthesis of TPP from thiamine. The reaction mixture contained 30 nmol of thiamine (or TMP), 2 μmol of adenosine 5'-triphosphate (ATP), 10 μmol of MgSO4, 100 μmol of Tris-hydrochloride (pH 7.5), and an aliquot of the enzyme preparation (3 mg of protein) in a total volume of 1.5 ml. To determine the activity in E. coli extracts, 15 μmol of KCl was added. The reaction was started by adding the enzyme preparation, incubated for 15 min at 37°C, and then terminated by heating the mixture for 5 min at 90°C after the pH was adjusted to 5 with 1.5 ml of 0.01 N HCl. After removal of denatured proteins by centrifugation, the TPP content was determined spectrophotometrically from the supernatant fluid by the method described previously (29).

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respectively, and they were enhanced with increasing incubation time. A small peak of \( R_f \) 0.31 was also tentatively identified as \([^{35}S]\)TMP (Fig. 2).

These results indicate that thiamine taken up by cells is readily converted to TPP in *M. denitrificans*. However, in *E. coli* it was recently reported that thiamine was transformed to TPP via TMP and that \([^{35}S]\)thiamine taken up appeared first in the radioactivity peak of \([^{35}S]\)TMP and later in the peak of \([^{35}S]\)TPP. Both the peaks corresponding to \([^{35}S]\)TMP and \([^{35}S]\)TPP increased with the incubation time (11). These findings suggest that TPP is synthesized from thiamine in *M. denitrificans* without involving the formation of TMP as an intermediate.

**Time course of TPP synthesis by extracts of *M. denitrificans*.** The time course of TPP synthesis from thiamine and TMP determined from the extracts of *M. denitrificans* is shown in Fig. 3. The synthesis of TPP from thiamine was much faster than that from TMP and reached an equilibrium at 10 min after incubation, whereas that from TMP proceeded linearly until 20 min after incubation.

It is interesting to note from the data presented here that TPP synthesis from thiamine through TMP by the successive two-step phosphorylation reactions does not take place in *M. denitrificans*. (The evidence supporting this assumption is given in Table 2: in *E. coli*, containing two enzymes which catalyze the formation of TPP via TMP, TMP was utilized as the substrate for TPP synthesis much better than thiamine.)

**Requirements for TPP synthesis.** Crude extracts of *M. denitrificans* were used to determine the requirements for TPP synthesis from thiamine (Table 1). The reaction was dependent on the presence of thiamine, ATP, and Mg\(^{2+}\). TMP was again found to be a much less effective substrate. ATP could be partially replaced with adenosine 5’-diphosphate (ADP), but this replacement did not occur with a partially purified enzyme preparation of this microorganism (data not shown).

**Effect of KCl on the activities of enzymes involved in TPP synthesis from thiamine.** In *E. coli* the synthesis of TPP from thiamine is dependent on the presence of K\. To distinguish the pathway of TPP synthesis in *M. denitrificans* from that in *E. coli*, the effect of K\( ^{+} \) on the activities of enzymes involved in TPP synthesis was determined (Table 2). With *E. coli* extracts, TPP synthesis from either thiamine or TMP was demonstrated only in the presence of K\( ^{+} \), and TMP was a better substrate than thiamine, which is consistent with the data of Nishino et al. (24). On the other hand, TPP synthesis by *M. denitrificans* extracts was independent of the presence of K\( ^{+} \) and was rather inhibited by K\. Thiamine was found to be a better substrate than TMP.

These results indicate that the formation of TPP from thiamine in *M. denitrificans* is catalyzed by an enzyme(s) different from that in *E. coli*.

![Fig. 2. Analysis of intracellular forms of \([^{35}S]\)thiamine transported. The cell suspension of *M. denitrificans* prepared as described in the text was incubated with 1 \(\mu M\) \([^{35}S]\)thiamine for 2 min (△) and 10 min (●) at 37 C. The fate of \([^{35}S]\)thiamine taken up by cells was analyzed by the paper chromatographic method described previously (11, 32). A solvent system of isopropyl alcohol-0.5 M sodium acetate buffer (pH 4.5)-water (65:15:20, vol/vol/vol) was used. In this solvent system thiamine, TMP, and TPP give \( R_f \) values of 0.64, 0.31, and 0.10, respectively (27).](Fig. 2)

![Fig. 3. Time course of TPP synthesis from thiamine (●) or TMP (△) by extracts of *M. denitrificans*. Extracts of *M. denitrificans* were obtained as described in the text; the reaction components are also given in the text. The amount of TPP synthesized from either thiamine or TMP was determined by the method described previously (29), in which TPP bound specifically with apoenzyme of yeast pyruvate decarboxylase (EC 4.1.1.1) can be measured by a decrease in the absorbancy at 340 nm by coupling to alcohol dehydrogenase reaction.](Fig. 3)
It should be effective substrate than the substrate mine, with analyzed found to be once to changed for 15 incubated tracts for M. denitrificans of 1.5 ml. umol of TPP reaction mixture. The enzyme, obtained from thiamine before synthesis was added to the reaction mixture containing 100 µmol of Tris-hydrochloride (pH 7.5), 30 nmol of thiamine, 2 µmol of ATP, 10 µmol of MgSO4, and dialyzed crude extracts (3 mg of protein) in a total volume of 1.5 ml. Thiamine or ATP was replaced with either 30 nmol of TMP or 2 µmol of ADP when necessary. Crude extracts (20 ml) were dialyzed for 8 h at 4 C against 3 liters of 50 mM Tris-hydrochloride (pH 7.5)-5 mM 2-mercaptoethanol (the medium was changed once after 4 h). The reaction mixtures were incubated for 15 min at 37 C, and the TPP formed was determined spectrophotometrically.

Boiled enzyme, obtained by treating crude extracts for 5 min in a boiling-water bath, was added to the complete system instead of native enzyme.

**Table 1. Requirements for the reaction of TPP synthesis by M. denitrificans extracts**

<table>
<thead>
<tr>
<th>Addition</th>
<th>TPP formed (nmol/mg of protein per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete*</td>
<td>0.572</td>
</tr>
<tr>
<td>-Thiamine</td>
<td>0</td>
</tr>
<tr>
<td>-Thiamine + TMP</td>
<td>0.074</td>
</tr>
<tr>
<td>-ATP</td>
<td>0</td>
</tr>
<tr>
<td>-ATP + ADP</td>
<td>0.240</td>
</tr>
<tr>
<td>-Mg2+</td>
<td>0</td>
</tr>
<tr>
<td>+Boiled enzyme*</td>
<td>0</td>
</tr>
</tbody>
</table>

* The complete reaction mixture contained: 100 µmol of Tris-hydrochloride (pH 7.5), 30 nmol of thiamine, 2 µmol of ATP, 10 µmol of MgSO4, and dialyzed crude extracts (3 mg of protein) in a total volume of 1.5 ml. Thiamine or ATP was replaced with either 30 nmol of TMP or 2 µmol of ADP when necessary. Crude extracts (20 ml) were dialyzed for 8 h at 4 C against 3 liters of 50 mM Tris-hydrochloride (pH 7.5)-5 mM 2-mercaptoethanol (the medium was changed once after 4 h). The reaction mixtures were incubated for 15 min at 37 C, and the TPP formed was determined spectrophotometrically.

**Table 2. Effect of K+ on the activities of enzymes involved in TPP synthesis from thiamine**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>TPP formed (nmol/mg of protein per h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-KCl</td>
</tr>
<tr>
<td><em>E. coli</em> K-12*</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>0</td>
</tr>
<tr>
<td>TMP</td>
<td>0</td>
</tr>
<tr>
<td><em>M. denitrificans</em></td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>0.582</td>
</tr>
<tr>
<td>TMP</td>
<td>0.259</td>
</tr>
</tbody>
</table>

* Potassium chloride (150 µmol) was added to the reaction mixture. The other conditions for the assay of TPP formed were identical to those described in Table 1.

* Sonic extracts of *E. coli* K-12 were prepared as described in the text and added to the reaction mixture instead of *M. denitrificans* extracts in the same amount of protein as that of the latter.

**Hydrolysis of TMP to thiamine by the crude extracts.** The fact that TMP is the much less effective substrate than thiamine (Fig. 3, Tables 1 and 2) suggests that TMP may be hydrolyzed once to thiamine before it is utilized as the substrate for TPP synthesis. TPP was found to be hydrolyzed to thiamine by the extracts at a much faster rate than TPP (Fig. 4). It should be noted that the specific activity (nanomoles per milligram of protein per hour) of the TMP-hydrolyzing enzyme was higher than that of the enzyme catalyzing the formation of TPP from thiamine (Table 1), indicating that TMP is readily hydrolyzed to thiamine to be used as the substrate for TPP synthesis.

**TTP synthesis by a partially purified preparation of *M. denitrificans*.** If TMP is utilized for TPP synthesis after hydrolysis to thiamine, removal of the TMP-hydrolyzing enzyme from the extracts may result in utilization of thiamine but not TMP. To test this, a partially purified preparation of the sonic extracts of *M. denitrificans* was obtained by the conventional purification method using ammonium sulfate fractionation and Sephadex and diethylaminoethyl-cellulose column chromatography. This preparation showed no activity of TPP-hydrolyzing enzyme and could catalyze TPP formation only from thiamine, whereas TMP was an inert substrate (H. Sanemori, manuscript in preparation).

These results suggest that the enzyme involved in the synthesis of TPP in *M. denitrificans* is similar to the thiamine pyrophosphokinase described in yeast. This assumption was supported by the direct synthesis of [³²S]TPP without formation of the intermediate TMP when [³²S]thiamine was incubated with the partially purified enzyme (H. Sanemori, manuscript in preparation). The enzyme catalyzing the direct synthesis of TPP from thiamine in *M. denitrificans* is referred to as thiamine pyrophosphokinase hereafter.

**De novo synthesis of TMP in *M. denitrificans*.** TMP is synthesized de novo from hydroxymethylpyrimidine and hydroxyethylthiazole.
in yeast (2-4, 14, 26-28), as well as in E. coli (10, 25), as shown in Fig. 1. Activities of the overall reaction and of the four enzymes involved in TMP synthesis were determined (Table 3). All of these activities were detected in extracts of M. denitrificans and were lower than those found in E. coli extracts. This suggests that a pathway of TMP synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole comparable to that in E. coli is operating in M. denitrificans.

Effect of adenosine added to the growth medium on TPP synthesis. It is important to determine whether or not a pathway of de novo TPP synthesis from TMP via thiamine is functioning physiologically in M. denitrificans. When adenosine or adenine is added to the growth medium, synthesis of the enzymes involved in TMP synthesis in E. coli can be derepressed, resulting in increases in the activities of these enzymes (9). With the extracts of M. denitrificans grown in the presence of 3 mM adenosine, activities of the overall reaction of TPP synthesis, thiaminephosphate pyrophosphorylase, TMP-phosphohydrolyzing enzyme, and thiamine pyrophosphokinase were measured (Table 4). All of these activities were found to be enhanced more than twofold in the presence of adenosine.

Adenine and adenosine were found to decrease the intracellular concentration of TPP (9, 22), which acts as a corepressor to bring about the regulation of thiamine biosynthesis. Therefore, the results described above suggest that the enzymes listed in Table 4 are involved in the physiological pathway of TPP synthesis in M. denitrificans.

TPP synthesis by cells of M. denitrificans grown anaerobically. M. denitrificans can grow well anaerobically on a medium containing NH4NO3 as the terminal electron acceptor instead of NH4Cl. Whether anaerobiosis of this organism induces an alternative pathway of TPP synthesis similar to that found in E. coli was also tested. Extracts of M. denitrificans cells grown either aerobically on NH4Cl or anaerobically on NH4NO3 showed almost identical formation of TPP from thiamine (0.495 and 0.552 nmol/mg of protein per h, respectively) and utilized TMP as a far less effective substrate than thiamine. Thus, the pathway of TPP synthesis in M. denitrificans was not altered for growth under aerobic and anaerobic conditions.

### DISCUSSION

The pathway of enzymatic synthesis of TPP, a key coenzyme in various enzyme reactions, is fairly well understood in E. coli (8, 12, 20-25) and yeast (for a review see reference 1). In E. coli, TPP is formed directly from TMP by de novo synthesis, whereas in yeast TPP formation takes place through the intermediate production of the free form of thiamine. In the present study we present evidence that there exist differences in TPP formation in E. coli and M. denitrificans, and the enzymatic sequence of TPP formation in M. denitrificans follows the same pathway as that elucidated for yeast.

The data that support the direct formation of TPP from thiamine are as follows. (i) [35S]Thiamine incubated with M. denitrificans cells was detected in the cells of [35S]TPPP or [35S]thiamine but not as [35S]TMP (Fig. 2). (ii) The rate of TPP synthesis was much faster from thiamine than from TMP when determined with the extracts (Fig. 3, Tables 1, 2, and 4). (iii) A partially purified preparation of M. denitrificans extracts, which does not contain the activity of TMP-phosphohydrolyzing enzyme,

### Table 3. Activities of enzymes involved in TMP synthesis

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Overall reaction</th>
<th>Enzyme activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>II</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>0.086</td>
<td>0.083</td>
</tr>
<tr>
<td>M. denitrificans</td>
<td>0.025</td>
<td>0.027</td>
</tr>
</tbody>
</table>

* The overall reaction of thiamine synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole (25) and the activities of four enzymes involved in TMP synthesis were determined with crude extracts of E. coli K-12 and M. denitrificans as described previously (9, 10). They are given as nanomoles per milligram of protein per hour.

* Enzymes I through IV correspond to those illustrated in Fig. 1. I, Hydroxymethylpyrimidine kinase; II, hydroxymethylpyrimidine phosphate kinase; III, hydroxyethylthiazole kinase; IV, thiaminephosphate pyrophosphorylase.

### Table 4. Effect of adenosine added to the growth medium on TPP synthesis in M. denitrificans

<table>
<thead>
<tr>
<th>Addition</th>
<th>Overall reaction</th>
<th>TMP pyrophosphorylase</th>
<th>TMP phosphatase</th>
<th>Thiamine pyrophosphokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.029</td>
<td>0.132</td>
<td>26.1</td>
<td>0.673</td>
</tr>
<tr>
<td>3 mM adenine</td>
<td>0.100</td>
<td>0.418</td>
<td>44.3</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* M. denitrificans was grown in a minimal medium (5) supplemented with or without 3 mM adenosine, and crude extracts were prepared as described in the text. The assay procedures for the enzyme activities, as well as the overall reaction of TPP synthesis, were as described in the text. Values given are nanomoles per milligram of protein per hour.
can convert only thiamine to TPP but not TMP to TPP and also can catalyze the formation of \([^{35}S]TPP\) from \([^{35}S]thiamine\) without forming \([^{35}S]TMP\) (H. Sanemori, manuscript in preparation). This evidence clarifies the point that the TPP synthesis from thiamine proceeds by a one-step pyrophosphorylation reaction without intermediate formation of TMP, like found in yeast but not in \(E. coli\). In addition to this, TPP synthesis from thiamine by \(E. coli\) extracts showed a complete dependence on \(K^+\), whereas in the case of \(M. denitrificans\) \(K^+\) partially inhibited TPP synthesis (Table 2). This indicates clearly that the enzyme system for TPP synthesis from thiamine in \(M. denitrificans\) is different from that in \(E. coli\).

De novo TMP synthesis from hydroxymethylpyrimidine and hydroxyethylthiazole in \(M. denitrificans\) occurs by the same pathway as in \(E. coli\) (10, 25) and yeast (for a review, see reference 1). The four enzymes involved in TMP synthesis are hydroxymethylpyrimidine kinase, hydroxymethylpyrimidine phosphate kinase, hydroxyethylthiazole kinase, and thiaminephosphate pyrophosphorylase, as illustrated in Fig. 1. TMP synthesized de novo in \(M. denitrificans\) should be hydrolyzed to thiamine by a phosphatase before conversion to the enzyme TPP; its formation is catalyzed by thiamine pyrophosphokinase as described above. If such a phosphatase specific for TMP, which is referred to as TMP phosphatase, is present and involved in de novo TPP synthesis from TMP via thiamine, the phosphatase should be regulated by the same mechanism that controls the other enzymes in the pathway. Adenosine, which causes derepression of thiamine biosynthesis when added to the growth medium (22), resulted in an increase in the activities of TPP-synthesizing enzymes (Table 4). TMP phosphatase was found to be one of these enzymes, and it was also evident that this TMP phosphatase enzyme was different from other phosphatases. In an preliminary experiment, a protein peak exhibiting the activity of TMP hydrolysis at pH 6.0 was separately obtained from the peak of acid phosphatase by Sephadex G-200 column chromatography (data not shown). In yeast (see reference 26 in review 1) and parsley leaf (19), which contains the same pathway for TPP synthesis as \(M. denitrificans\), it was demonstrated that TMP synthesized de novo is hydrolyzed to thiamine by phosphatases, including acid phosphatase with broad substrate specificities.

The results described above indicate the presence of a possible pathway of de novo TPP synthesis in \(M. denitrificans\), as illustrated in Fig. 1. The reason why \(E. coli\) and \(M. denitrificans\) contain different pathways for TPP synthesis from thiamine has not yet been elucidated. A toxonomic survey of this pathway is now under way.

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LITERATURE CITED