Bacterial Metabolism of Thiamine

III. METABOLISM OF THIAMINE TO 3-(2'-METHYL-4'-AMINO-5'-PYRIMIDYLMETHYL)-4-METHYLTIAZOLE-5-ACETIC ACID (THIAMINE ACETIC ACID) BY A FLAVOPROTEIN ISOLATED FROM A SOIL MICROORGANISM*

ROBERT A. NEAL

From the Department of Biochemistry, Division of Nutrition, Vanderbilt University School of Medicine, Nashville, Tennessee 37203

SUMMARY

A flavoprotein which catalyzes the metabolism of thiamine to 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid (thiamine acetic acid) has been purified to homogeneity. The purification and certain properties of the enzyme are described. The enzyme catalyzes a reaction in which 1 mole of thiamine and 2 moles of oxygen are consumed to form 1 mole of thiamine acetic acid. The evidence reported here suggests that thiamine is oxidized to thiamine acetic acid by a single enzyme without the intermediate aldehyde being released from the enzyme. The enzyme is active against thiamine, oxythiamine, and pyrithiamine but not thiochrome. At an equimolar concentration, thiamine acetic acid decreases the rate of oxidation of thiamine by 40%. The enzyme is inhibited by p-chloromercuribenzoate, quinacrine, semicarbazide, Hg^2+, and Cu^2+ and stimulated by mercaptoethanol, NaCN, and EDTA.

EXPERIMENTAL PROCEDURE

Materials—All chemicals were the purest commercial grade available. Glass-redistilled water was used in all experiments. Tris, sodium phosphate, and magnesium EDTA (Fisher) were used for buffers. Enzyme grade (NH_4)_2SO_4 (Mann) was used. All of the cross-linked dextran gels were purchased from Pharmacia. Microcrystalline DEAE-cellulose was obtained from Reeve Angel. Amberlite CG-50 was a product of Mallinkrodt. Thiamine-HCl was purchased from Merck. Oxythiamine and pyrithiamine were obtained from Nutritional Biochemicals. Protamine sulfate, phenazine methosulfate, and nitro blue tetrazolium were purchased from Sigma. NAD, NADP, NADPH, NADH, FAD, FMN, and catalase were obtained from Boehringer Mannheim. Aniline blue-black and other materials used for disc electrophoresis were products of Canalco.

Miscellaneous Methods—Protein samples were concentrated at 4°C either by use of an Amicon Diaflo ultrafiltration apparatus or by lyophilization.

Polyacrylamido gel electrophoresis was performed in a disc electrophoresis apparatus at room temperature with the use of columns, 0.5 x 6 cm, at 50 mg per column. Previously run gels were stacked at pH 8.9 with 100 to 400 mg of protein and run at pH 9.5. Gels were stained for protein by immersing them in a solution of 0.5% aniline blue-black in 7.0% acetic acid for 1 hour. The gels were destained overnight in 10% acetic acid.

The protein bands corresponding to thiamine dehydrogenase were detected by immersing the extruded gel in sodium phosphate buffer (50 mM, pH 7.0) containing 0.004% phenazine methosulfate, 0.040% nitro blue tetrazolium, and 0.015% thiamine. As soon as the blue bands corresponding to thiamine dehydrogenase were clearly visible (usually 1 to 2 min), the gel was removed from the solution and washed with water and the protein bands were fixed by placing the gel in 10% trichloroacetic acid for 30 min. The gels were stored in 5% acetic acid.

Protein was estimated by the method of Lowry et al. (3) with bovine serum albumin (Armour) as a standard. The method of Warburg and Christian (4) was used to assay the protein in the column fractions.

The differential fluorometric method of Wilson and King (5) was used in an attempt to determine the stoichiometry of the binding of the flavin nucleotide to the apoenzyme. Iron was determined by the method of Seven and Peterson (6) and molybdenum by the method of Johnson and Arkley (7).

The procedures used to grow and harvest the microorganism used in these studies have already been described (1).

Enzyme Assay Procedures—The activity of the enzyme was determined manometrically by measuring oxygen uptake or by following the reduction of ferricyanide in the presence of enzyme.

* This investigation was supported by United States Public Health Service Research Grant AM-10297.
and substrate. The ferricyanide reduction method showed considerable variability and, therefore, was not used for accurate quantitative measurements of enzyme activity. However, this assay procedure proved to be a rapid and convenient method for locating the thiamine dehydrogenase activity in the various column fractions during the purification procedures.

The ferricyanide assay was carried out with the use of a Cary model 15 spectrophotometer equipped with automatic sample changing. The dehydrogenase activity was measured by adding 0.05 to 0.2 ml of the enzyme solution to a 10-mm cuvette containing 100 μmoles of sodium phosphate (pH 7.0) and 10 μmoles of thiamine-HCl in a final volume of 1.8 ml. Ferricyanide (0.07 ml, 1.75 μmoles) was quickly added, the solution was mixed, and the rate of decrease in optical density at 420 nm was followed and compared with the rate observed in a cuvette containing buffer, thiamine, and ferricyanide but no enzyme. The rate of change of optical density of the blank averaged about 0.050 per min. As many as five such assays were conducted at the same time by recording the optical density of the samples sequentially for 10-sec intervals.

The enzyme could also catalyze the oxidation of thiamine with 2,6-dichlorindophenol or a coupled system of phenazine methosulfate and nitro blue tetrazolium as electron acceptors. Cytochrome c, NAD and NADP were not electron acceptors in this system.

The manometric assays were carried out in a Gilson respirometer with single side arm flasks. Thiamine chloride (usually 10 μmoles) was added to the side arm. The main chamber contained 100 μmoles of sodium phosphate (pH 7.2), 2.0 μmoles of EDTA, 0.2 μmole of mercaptoethanol (Buffer A), and 0.05 to 0.2 ml of enzyme in a final volume of 1.9 ml. The center well contained 0.2 ml of 30% sodium hydroxide. The contents of the main chamber were equilibrated for 10 min at 30°, the thiamine was added, and oxygen uptake was measured. In the studies of the effect of various inhibitors, metal chelators, and compounds containing sulfhydryl groups, the EDTA and mercaptoethanol were excluded.

**Determination of Absorption Spectra**—In order to show a decrease in the absorption spectra of the enzyme in the presence of thiamine it was necessary to take special precautions to remove oxygen from the enzyme solution. This was accomplished by placing the enzyme solution in a cuvette fitted with a side arm and a gas-tight cover which allowed for evacuation and for bubbling of a gas through the enzyme solution. The cuvette was first evacuated to 5-mm pressure with a vacuum pump. Next oxygen-free helium was bubbled through the solution for 30 min. The cuvette was again evacuated and the bubbling of oxygen-free helium through the solution was repeated. The spectrum of the enzyme following this treatment was considered to be the oxidized spectrum. Thiamine or sodium hydrosulfite was then added and the reduced spectrum was recorded.

**RESULTS**

**Purification of Enzyme**—Lyophilized cells were suspended in 50 mM Tris hydrochloride buffer (pH 8.5) containing 1 mM EDTA and 0.1 mM mercaptoethanol at a concentration of 10 g of cells per 150 ml. One hundred fifty-milliliter batches of the cell suspension were subjected to sonic vibration for 20 min in a Bronson W-185-C sonifier at a power setting of 85 watts. The temperature was maintained between 25 and 30° during the sonic disruption by means of an ice bath. All subsequent operations were carried out at 0-4°. Ten such sonic treatments were performed and the material was pooled and centrifuged at 15,000 × g for 20 min (Step 1, Table 1). Ammonium sulfate to 20% saturation was added to the cell-free extract and the solution was centrifuged. Thiamine dehydrogenase was precipitated from the resultant supernatant solution by adding ammonium sulfate to 80% saturation. The precipitate was dissolved in the Tris buffer, the solution was divided into four equal parts, and each portion placed on a column, 5 × 50 cm, of Sephadex G-100. The enzyme was eluted from the column with the same Tris buffer. The most active fractions from these four columns, as determined by the ferricyanide assay, were pooled (Step 2) and the pH was adjusted to 7.0 with 1 N hydrochloric acid. Thiamine sulfate was added with stirring at a concentration of 1 mg/10 mg of protein. After 30 min the solution was centrifuged and the pH of the supernatant fluid was adjusted to 8.5 with 5.0 N aqueous ammonia (Step 3). The protein concentration of the supernatant was adjusted to 10 mg per ml and the resultant solution was treated with ammonium sulfate from 0 to 45% saturation followed by 45 to 60% saturation. The precipitate from the 45 to 60% ammonium sulfate saturation was dissolved in the Tris buffer and the solution was desalted by repeated dilution and reduction of the volume on an Amicon Diaflo ultrafiltration apparatus (Step 4). The desalted solution from Step 4 was placed on a column, 0.9 × 10 cm, of microcrystalline DEAE-cellulose which had been equilibrated with the Tris buffer described previously but containing 0.05 M sodium chloride. The enzyme was eluted with a 0.05 to 0.25 M linear gradient of sodium chloride in 100 ml of the same buffer. The most active fractions from this column were pooled. A summary of the purification procedure is given in Table I. Disc gel electrophoresis of aliquots of Steps 1, 2, and 3 is shown in Fig. 1. As can be seen, the staining of the gels from Steps 1 and 2 as a result of enzyme activity (1b, 11b) reveals the presence of three proteins capable of oxidizing thiamine in the presence of phenazine methosulfate, nitro blue tetrazolium system as an electron acceptor. Protein staining of the gel from Step 5 (IIIa) reveals the presence of two proteins, both of which also stain for enzyme activity (IIIb). When the two proteins from gels analogous to IIIb were isolated separately and again subjected to disc gel electrophoresis, in each case both protein staining and enzymatic staining revealed a single band. Fig. 1, 1Vα and 1Vβ,
FIG. 1. Polyacrylamide gel electrophoresis of protein from the various steps in the purification of thiamine dehydrogenase (Table I). The sample gel was prepared by mixing 25 to 50 μl containing 100 to 400 μg of protein with a volume of gel solution sufficient to make a final volume of 200 μl. The gel was prepared as specified in the Canalco manual. The staining of the gel for protein and enzymatic activity is described under “Experimental Procedure.” la, Step 1, Table I, protein staining; Ib, Step 1, Table I, enzymatic staining; IIa, Step 2, Table I, protein staining; IIb, Step 2, Table I, enzymatic staining; IIIa, Step 5, Table I, protein staining; IIIb, Step 5, Table I, enzymatic staining; IVa, upper protein band; IVb, upper protein band; IIIb, enzymatic staining.

shows the results obtained when the upper protein in IIIb was isolated and again subjected to disc electrophoresis. When both proteins from IIIb were isolated from the gel and incubated separately with thiamine, the sole product was found to be thiamine acetic acid in either case. The specific activities of these two enzymes isolated from gel are considerably less than the specific activity of the Step 5 material applied to the gel. In all cases tested, the lower or minor component had a lower specific activity (approximately 0.45 unit) than the upper or major component (approximately 0.70 unit). When the two proteins were mixed the specific activity was the approximate average of the individual specific activities.

Thus the protein eluted from the DEAE-cellulose column (Step 5) appears to be homogeneous from an enzymatic standpoint. Whether the three proteins in Ia and IIb and the two protein components in IIIb represent isozymic forms of the enzyme or a structural modification of the enzyme during the first step in the isolation of the enzyme remains to be determined. The two lower proteins in Ia and IIb are apparently analogous to the two proteins in IIIb. The protein in the upper band in IIb also oxidizes thiamine to thiamine acetic acid, but has a lower specific activity than the protein in the two lower bands. This upper band protein is separated from the other active proteins on DEAE-cellulose.

Absorption Spectra of Enzyme—The absorption spectrum of the enzyme in Step 5 (Table I) in 50 mM Tris buffer (pH 8.5) shows a maxima at 368 and 444 μM, shoulders at 424 and 470 μM, and a minimum at 404 μM. This absorption spectrum is typical of known flavoproteins. When thiamine is added to the enzyme in a molar ratio of 5 moles of thiamine per mole of enzyme under anaerobic conditions, there is a decrease in absorption throughout the range of 350 to 500 μM (Curve B). The decrease in absorption is reversed on exposure of the solution of the enzyme to oxygen. There is also a reversible decrease in absorption of the enzyme on addition of sodium hydrosulfite (Curve C).

Molecular Weight, pH Optimum, and Michaelis Constant—The molecular weight of thiamine dehydrogenase was determined by the gel filtration method of Andrews (8) on a column, 2.5 × 90.0 cm, of Sephadex G-100 (Superfine) which was developed by the downward flow technique. After equilibration of the column with 100 mM sodium phosphate buffer, (pH 7.2), a 1-ml sample containing 2 to 5 mg each of blue dextran, ovalbumin, bovine serum albumin, chymotrypsinogen, myoglobin, and 0.5 mg of thiamine dehydrogenase was pumped onto the column and washed through at a flow rate of 6.0 ml per hour at 4°. Fractions of 2 ml each were collected and their absorbances at 280 μM were determined. The molecular weight of thiamine dehydrogenase was determined by plotting the ratios of the elution volume (Ve) to the void volume (Vv) against the logarithm of the molecular weight of the proteins tested (9). By the use of this technique, the molecular weight of thiamine dehydrogenase was calculated to be 41,500.

The enzyme was active over a wide pH range with maximal activity occurring between pH 8.0 and 9.0. Since thiamine is unstable above pH 8.0, most of the experiments described in this paper were carried out at pH 7.2 in phosphate buffer. This
Stoichiometry of the oxidation of thiamine in the presence of thiamine dehydrogenase

Thiazole-2-\(^{13}\)C-thiamine (0.5 \(\mu\)Ci per \(\mu\)mole) in the quantities indicated was incubated in duplicate in a Gilson respirometer with thiamine dehydrogenase. The incubation mixture contained thiamine; enzyme (10 \(\mu\)g); sodium phosphate, pH 7.2, 100 \(\mu\)moles; mercaptoethanol, 0.2 \(\mu\)mole; EDTA, 2.0 \(\mu\)mole, in a final volume of 2.0 ml. The incubations were continued until no more oxygen was taken up (3 hours for 1 \(\mu\)mole of thiamine, 6 hours for 5 \(\mu\)moles). The duplicates at each substrate level were pooled and chromographed on an Amberlite CG-50 column, 1 \(\times\) 30 cm (2). The \(^{13}\)C-thiamine acetic acid formed during the incubation and the \(^{14}\)C-thiamine remaining at the end of the incubation were determined by pooling the column fractions containing these compounds and determining the radioactivity by scintillation counting. The enzyme used in these and all subsequent experiments in this report was equivalent to the enzyme in Step 5, Table I, and contains two forms of thiamine dehydrogenase.

<table>
<thead>
<tr>
<th>Initial thiamine</th>
<th>Final thiamine</th>
<th>Thiamine acetic acid formed</th>
<th>(\mu)moles</th>
<th>(\mu)moles consumed</th>
<th>Thiamine metabolized</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.00)</td>
<td>None</td>
<td>0.08</td>
<td>1.95</td>
<td>1.95</td>
<td>1.95</td>
</tr>
<tr>
<td>(2.00)</td>
<td>0.03</td>
<td>1.94</td>
<td>3.88</td>
<td>1.97</td>
<td>1.97</td>
</tr>
<tr>
<td>(3.00)</td>
<td>0.17</td>
<td>2.77</td>
<td>5.40</td>
<td>1.91</td>
<td>1.91</td>
</tr>
<tr>
<td>(4.00)</td>
<td>0.36</td>
<td>3.36</td>
<td>6.55</td>
<td>1.80</td>
<td>1.80</td>
</tr>
<tr>
<td>(5.00)</td>
<td>0.86</td>
<td>4.03</td>
<td>8.09</td>
<td>1.93</td>
<td>1.93</td>
</tr>
</tbody>
</table>

was the best compromise between substrate stability and enzyme activity.

With thiamine as the substrate the Michaelis constant of thiamine dehydrogenase was determined manometrically. A \(K_m\) value of 1.72 mM was determined mathematically, the location of the “best straight line” being calculated by the method of least squares. By use of the manometric assay, the catalytic constant for the enzyme was determined to be approximately 100 (moles of product formed per min per mole of enzyme).

The enzyme can be stored at \(-70^\circ\) for as long as 3 months and at \(4^\circ\) for as long as 1 week without appreciable loss in activity. The enzyme is stable at 50\(^\circ\) for 30 min. At 60\(^\circ\) there is a 30\% loss in activity in 30 min. All activity is lost when a solution of the enzyme is heated above 65\(^\circ\) for 5 min. The heat stability of the enzyme appears to be independent of the protein concentration.

Flavin, Molybdenum, and Iron Content of Enzyme—By use of the fluorometric method of Wilson and King (5), the dissociation of the flavin from the enzyme by conventional methods such as heating to boiling at neutral pH, extraction with 10\% trichloroacetic acid at 0\(^\circ\), or treatment with 6 \(\mu\)M guanidine could not be detected. These data indicate that the flavin moiety may be covalently bound to the enzyme. Spectrophotometric determination of the flavin nucleotide content of the enzyme with an extinction coefficient of 11.3 cm\(^2\) \(\mu\)mole\(^{-1}\) indicates 0.37 mole of flavin per 41,500 g of protein. The less than 1:1 stoichiometry of flavin to apoenzyme could be the result of partial dissociation of the flavin from the enzyme, an inaccurate estimation of the molecular weight of the enzyme, or the extinction coefficient of the bound being less than that of the free flavin nucleotide. Further work will be required to determine which of the alternatives is correct.

The enzyme was also examined for its content of iron (6) and molybdenum (7). No molybdenum could be detected and only a trace (less than 0.05 mole per mole of enzyme) of iron was present.

Stoichiometry of Reaction—The stoichiometry of the oxidation of thiamine by thiamine dehydrogenase is shown in Table II. The data indicate that 2 \(\mu\)moles of oxygen are consumed and 1 \(\mu\)mole of thiamine acetic acid is formed per \(\mu\)mole of thiamine metabolized. Analysis of the reaction mixtures by ion exchange and thin layer chromatography (2) revealed that thiamine and thiamine acetic acid were the only radioactive constituents in the reaction mixture at the end of the incubation. Therefore, these data also show that oxygen uptake is an accurate measure of the enzyme activity.

Substrate Specificity—The compounds oxythiamine and pyri-thiamine were oxidized at a rate which was 77 and 58\% that of thiamine. The three principal thiamine metabolites which have a primary hydroxyl group in their structure (5-(2-hydroxyethyl) -4-methylthiazole, 2-methyl-4-amino-5-hydroxymethylpyrimidine, 2-methyl-4-hydroxy-5-hydroxymethylpyrimidine) (2), as well as thiochrome and ethanol, were not metabolized by this enzyme at a detectable rate.

The oxidation products resulting from the incubation of oxythiamine and pyri-thiamine with thiamine dehydrogenase were examined by thin layer chromatography. An aliquot of each reaction mixture was spotted and the plates were developed with \(\alpha\)-propyl alcohol-acetate buffer (1 \(\mu\), pH 3.0)-water (70:10: 20, v/v). Both chromatograms showed unmetabolized substrate plus a second metabolite with a lower \(R_F\) value (\(R_F\) oxythiamine = 0.51, pyri-thiamine = 0.55, oxythiamine oxidation product = 0.43, pyri-thiamine oxidation product = 0.50). The oxythiamine oxidation product was eluted from the cellulose and lyophilized, and the residue was dissolved up in 1 ml of 1 M sodium bisulfite. After 24 hours at room temperature the sodium bisulfite solution was extracted twice with 3 ml of ethyl acetate and the ethyl acetate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.1 ml of 50\% ethanol and spotted on a thin layer of cellulose along with the standards 4-methylthiazole-5-acetic acid, 5-(2-hydroxyethyl)-4-methylthiazole, and 2-methyl-4-hydroxy-5-pyrindymethyl-sulfonic acid. The chromatogram was developed with chloroform-methanol-3.5 \(\%\) aqueous \(\text{NH}_2\) \(\times\) (55:45:3.5, v/v). When the chromatogram was examined under ultraviolet light, only two compounds were visible. One of these compounds had an \(R_F\) value identical with 4-methylthiazole-5-acetic acid (0.55). The other compound had an \(R_F\) identical with 2-methyl-4-hydroxy-5-pyrindymethyl-sulfonic acid (0.19). Thus, as in the case of thiamine, the site of oxidation of oxythiamine is the hydroxymethyl side chain of the thiazole moiety. The nature of the oxidation product of pyri-thiamine was not further examined. Its behavior on thin layers of cellulose suggests, however, that the site of oxidation of this compound is the hydroxymethyl side of the pyridine moiety.

End Product Inhibition—Thiamine dehydrogenase was previously incubated with Buffer A for 10 min. Thiamine and thiamine acetic acid were then added and the rate of oxygen uptake was determined. The results are shown in Table III. As the concentration of thiamine acetic acid approached 0.75 mM in the reaction mixture, the rate of oxidation of thiamine decreased, although thiamine in amounts sufficient to saturate the enzyme was present. The inhibition by thiamine acetic acid was evident within 5 min after the addition of the mixture of
that thiamine acetic acid had in some way protected the enzyme.

Semicarbazide had a similar effect. When thiamine acetic acid was inactivating the enzyme, thiamine dehydrogenase uptake approached 40%. To determine whether thiamine concentration of thiamine acetic acid was increased until it was

During the incubation and subsequent dialysis, the loss in thiamine was determined. The results of two separate experiments showed that the incubation which had contained thiamine was approximately 30% more active after dialysis than the one which had contained thiamine. It thus appears that thiamine acetic acid had in some way protected the enzyme during the incubation and subsequent dialysis. The loss in activity of thiamine dehydrogenase upon dialysis could not be restored with FAD, FMN, or a combination of these two coen-

\[ \text{Relative activity} = \frac{\text{Activity after dialysis}}{\text{Activity before dialysis}} \]

<table>
<thead>
<tr>
<th>Concentration of thiamine acetic acid</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>0.38</td>
<td>100</td>
</tr>
<tr>
<td>0.75</td>
<td>90</td>
</tr>
<tr>
<td>1.5</td>
<td>82</td>
</tr>
<tr>
<td>3.0</td>
<td>74</td>
</tr>
<tr>
<td>5.0</td>
<td>59</td>
</tr>
</tbody>
</table>

**Table IV**

**Effect of various compounds on enzyme activity**

The incubation mixture contained 100 µmoles of sodium phosphate (pH 7.2), enzyme (5 µg per ml), and the various compounds in a final volume of 1.9 ml. The thiamine dehydrogenase was previously incubated with the various compounds for 10 min, thiamine (10 µmoles) was added, and the incubation was carried out for 60 min. The oxygen uptake was measured for 60 min after the addition of thiamine, in the incubation containing thiamine only was assigned a value of 100. The final concentration of thiamine in each incubation was 5.0 mM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Quinacrine</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>NaCN</td>
<td>1</td>
<td>121</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>117</td>
</tr>
<tr>
<td>Semicarbazide</td>
<td>10</td>
<td>66</td>
</tr>
<tr>
<td>p-Chloromercuri-benzoate</td>
<td>0.5</td>
<td>24</td>
</tr>
<tr>
<td>p-Chloromercuri-benzoate</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>2.0</td>
<td>180</td>
</tr>
<tr>
<td>Mereaptotoxahe</td>
<td>6.0</td>
<td>171</td>
</tr>
<tr>
<td>Gluthione (reduced)</td>
<td>1.5</td>
<td>171</td>
</tr>
<tr>
<td>HgCl2</td>
<td>0.05</td>
<td>14</td>
</tr>
<tr>
<td>CuCl2</td>
<td>0.05</td>
<td>81</td>
</tr>
<tr>
<td>CuCl2</td>
<td>0.05</td>
<td>12</td>
</tr>
<tr>
<td>CuCl2</td>
<td>0.05</td>
<td>81</td>
</tr>
</tbody>
</table>

**Table V**

**Reversibility of thiamine dehydrogenase inhibition by semicarbazide and CuCl2**

Thiamine dehydrogenase was incubated with the inhibitor, thiamine, and inhibitor plus thiamine, as described in Table IV. After the incubation the reaction mixtures were dialyzed for 3 hours against 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA. The incubation mixtures were then tested for their ability to oxidize thiamine. The rate of oxidation by the enzyme, which was incubated with thiamine only, was assigned a value of 100 both before and after dialysis.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Before dialysis</th>
<th>After dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine (5.00 mM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Thiamine (5.00 mM) + CuCl2 (0.05 mM)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Thiamine (5.00 mM) + semicarbazide (10 mM) + CuCl2 (0.05 mM)</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Thiamine (5.00 mM) + catalase (10 units) + NaCN (1 mM)</td>
<td>133</td>
<td>133</td>
</tr>
</tbody>
</table>

There is no evidence of substrate inhibition with thiamine concentrations up to 25 mM (15 times the \( K_a \)).

**Effect of Various Compounds on Enzyme Activity**—The effect of various compounds on the rate of oxidation of thiamine by thiamine dehydrogenase is shown in Table IV. In addition to these compounds, Mg2+, Mn2+, Cu2+, MoO42-, FAD, FMN, NAD, NADH, NADP, NADPH, and combinations of these compounds were examined and found to have no effect. In addition 1,10-phenanthroline (5 mM), iodoacetamide (1 mM), ethanolic (8.5 x 10⁻¹ M), 8-hydroxyquinoline (5 mM), bathophenanthroline (5 mM), menadione (10 µM), and Zn2+, Pb2+, Fe3+, Cu2+, all at 1 mM, had neither an inhibitory nor a stimulatory effect on the enzyme. The stimulatory effect of the thiol compounds, dithiothreitol, mercaptoethanol, glutathione, and the inhibition of the enzyme by p-chloromercuribenzoate, Hg2+, and Cu2+ suggest the presence of a sulfhydryl group on the enzyme which is essential for activity.

The only other compounds which are significantly inhibitory are quinacrine and semicarbazide. The inhibitory effect of quinacrine has not been examined in any detail. Further experiments will be required to show whether quinacrine inhibits by interference with the function of flavin on the enzyme.

The incubation mixtures containing semicarbazide and CuCl2 were dialyzed to see whether the inhibition was reversible. The results are shown in Table V. The inhibition by CuCl2 in
the presence and absence of substrate was not reversible by dialysis, whereas the semicarbazide and semicarbazide plus thiamine enzyme had a greater activity than the control after dialysis. The control enzyme lost about 30% of its activity on dialysis. The semicarbazide and semicarbazide plus thiamine inhibited mixture after dialysis had about 99 and 62%, respectively, of the activity of the control enzyme before dialysis. Thus, semicarbazide seems to protect the enzyme from inactivation during incubation and subsequent dialysis but the protective effect of semicarbazide does not require the presence of substrate.

The presence of flavin in this enzyme indicates that the end product of the reaction could be $\text{H}_2\text{O}_2$. If the enzyme preparation had some contaminating hydroperoxidase activity, the $\text{H}_2\text{O}_2$ would be decomposed to $\text{H}_2\text{O}$ and $\text{O}_2$, and the stimulatory effect of cyanide could be the result of an inhibition of the peroxidase activity with an increase in the apparent oxygen consumption. The effect of catalase on the rate of oxygen uptake catalyzed by thiamine dehydrogenase is shown in Table VI. The data indicate that the rate of oxygen uptake is decreased in the presence of catalase and that cyanide will reverse the decrease in oxygen uptake brought about by catalase. The enzyme preparation was examined manometrically for its ability to release $\text{O}_2$ from $\text{H}_2\text{O}_2$ and was found to be inactive. It thus appears the stimulatory effect of cyanide must be for a reason other than inhibition of peroxidase activity. Alternative explanations could be a combination of the cyanide ion with heavy metals such as $\text{Cu}^{2+}$ or $\text{Hg}^{2+}$ present in trace amounts in the incubation media. The cyanide may also be acting to reduce a disulfide linkage formed by the action of $\text{H}_2\text{O}_2$ on neighboring sulfhydryl groups.

The stimulatory effect of EDTA could also be attributable to a binding of heavy metals. An alternate explanation could be an involvement of the added EDTA in a photochemical reduction of flavin moiety on the enzyme.

The ability of thiamine dehydrogenase to catalyze the oxidation of NADPH and NADH with oxygen or ferricyanide as electron acceptors was examined both manometrically (oxygen uptake) and spectrophotometrically by following the decrease in absorbance at 340 nm in the presence and absence of ferrocyanide. The enzyme was not able to catalyze the oxidation of either of these two compounds. In addition, the enzyme is not able to reduce NAD$^+$ and NADP$^+$ with thiamine as a substrate.

**DISCUSSION**

Since the oxidation of thiamine to thiamine acetic acid requires 2 moles of oxygen, a two-step reaction is implied. The intermediate in the reaction should be thiamine aldehyde. Attempts at detecting the accumulation of this intermediate in the reaction mixture have not been successful.

If the inhibitory effect of semicarbazide is a result of its combining with the intermediate aldehyde and preventing its further oxidation, it might be expected that an additional compound other than thiamine and thiamine acetic acid might be detectable in an incubation medium containing thiamine, enzyme, and semicarbazide. Again, no compound other than thiamine and thiamine acetic acid could be detected.

Attempts at synthesizing 3-(2'-methyl-4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetaldehyde (thiamine aldehyde) for testing as a substrate for the enzyme have, so far, not been successful. Oxidizing thiamine directly with $\text{Cr}_2\text{O}_7$ in numerous solvents leads to a mixture of products, none of which is reactive with 2,4-dinitrophenylhydrazine to give a hydrazone. The intermediate thiazole aldehyde, 4-methylthiazole-5-acetaldehyde, is easily synthesized by adding $\text{Cr}_2\text{O}_7$ in t-butyl alcohol to a solution of 5-(2-hydroxyethyl)-4-methylthiazole in the same solvent. Attempts at coupling this aldehyde with the pyrimidine moiety of thiamine again led to a mixture of products, none of which is capable of forming a 2,4-dinitrophenylhydrazone. The compound 4-methylthiazole-5-acetaldehyde is not oxidized by the enzyme. This is not unexpected, however, since the corresponding alcohol 5-(2-hydroxyethyl)-4-methylthiazole is also not a substrate for thiamine dehydrogenase.

The apparent enzymatic homogeneity of the purified enzyme and the failure to detect any intermediate aldehyde in the reaction mixture indicate that the two-step oxidation of thiamine to thiamine acetic acid is being catalyzed by a single enzyme according to the following equation:

$$\text{Thiamine} + 2\text{O}_2 \rightarrow \text{thiamine acetic acid} + 2\text{H}_2\text{O}_2$$

The enzyme histidinol dehydrogenase catalyzes the two-step oxidation of histidinol to histidine (10). The intermediate histidinal has not been isolated as an intermediate but has been shown to be oxidized by this enzyme to histidine. In this reaction, 2 moles of NAD$^+$ are reduced for every mole of substrate oxidized. Several other enzymes which catalyze two sequential pyridine nucleotide-linked dehydrogenation reactions have been reported (11-14), but this is the first report of a flavoprotein catalyzing a similar two-step oxidation in which the intermediate in the oxidation is not released from the enzyme. Xanthine oxidase catalyzes the oxidation of hypoxanthine to uric acid. Although this is a two-step oxidation, the intermediate compound xanthine is released from the enzyme during the reaction (15).

**Acknowledgments**—The expert technical assistance of Mrs. Shirley Welch, Mrs. Helen Brown, and Mr. Kwong Sui Ling is gratefully acknowledged.

**REFERENCES**

Bacterial Metabolism of Thiamine: III. METABOLISM OF THIAMINE TO 3-(2'-METHYL-4'-AMINO-5'-PYRIMIDYLMETHYL)-4-METHYLTHIAZOLE-5-ACETIC ACID (THIAMINE ACETIC ACID) BY A FLAVOPROTEIN ISOLATED FROM A SOIL MICROORGANISM

Robert A. Neal


Access the most updated version of this article at http://www.jbc.org/content/245/10/2599

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/10/2599.full.html#ref-list-1