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ENZYMATIC REDUCTION OF DISULFIDE-TYPE THIAMINE DERIVATIVES

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Abstract—An enzyme which catalyzed the thiol–disulfide exchange reaction between reduced glutathione and disulfide-type thiamine derivatives, a newly developed fat soluble thiamine derivative, was described. Enzyme from rat liver was partially purified and its substrate specificity was studied. Reduced glutathione was an active hydrogen donor and other thiols and reducing compounds tested were relatively poor cofactor for the enzyme. This enzyme was specific only for disulfide-type thiamine derivatives and seemed to be different from enzymes which have been reported to catalyze thiol–disulfide exchange reaction.

IN RECENT years, there have been many developments in research of modified thiamine compounds and a large number of reports concerned with so-called fat soluble thiamine published in Japan.[1] Disulfide-type thiamine analogues are widely used in the clinical field because of good absorption and their persistence at high concentrations in the blood. Metabolic studies on the thiamine derivatives have so far given little information concerning the enzyme systems involved. Apparently, scission of the disulfide bond of the thiamine derivatives is one of the important steps in their metabolic conversion as well as their thiamine activity *in vivo*. The mechanism of the disulfide bond cleavage was reported to be a nonenzymatic thiol–disulfide exchange reaction with SH groups of glutathione, hemoglobin and plasma albumin (Fig. 1),

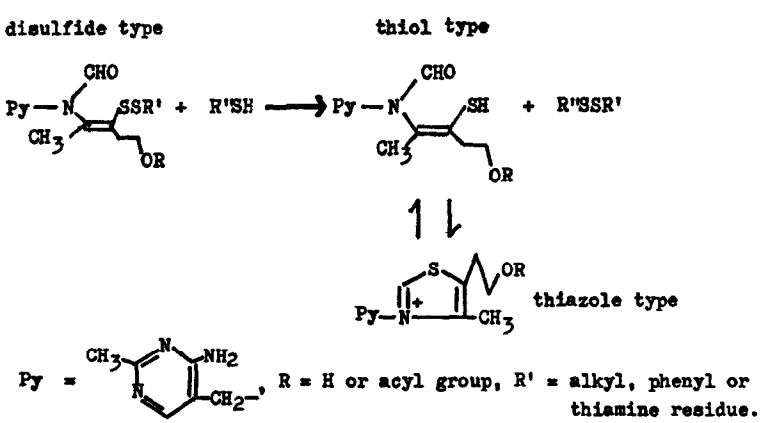

FIG. 1. Reduction of disulfide-type thiamine derivatives by thiol groups.

especially in blood, in which the metabolic pathways of the thiamine derivatives were frequently investigated.[2–9] When glutathione was the active species in the exchange reaction, the regeneration of reduced glutathione by glutathione reductase (EC 1.6.4.2) occurred.[9–11] However, all attempts to demonstrate direct enzymatic cleavage of the analogue failed to produce clear evidence for the presence of a NAD(P)H-linked enzyme in rat liver.[12] In this paper, we present evidence for the enzymatic reduction of these disulfide-type thiamine derivatives in rat tissues. In homogenates of rat tissues, there exists a transhydrogenase-type enzyme which catalyzes the transfer of hydrogen from reduced glutathione to disulfide-type thiamine derivatives.

MATERIALS AND METHODS

Materials. Thiamine methyl disulfide (TMD), thiamine ethyl disulfide (TED), thiamine propyl disulfide (TPD), thiamine butyl disulfide (TBD) were prepared by the method of Matsukawa.[13] Thiamine phenyl disulfide (TPhD) was prepared as previously described.[14] *O*-Benzoyl thiamine propyl disulfide (BTPD) and *O*-butyryl thiamine propyl disulfide (BuTPD) were prepared according to Kawasaki.[15] Reduced glutathione (GSH) was the product of Tanabe Seiyaku Co., Ltd. NADPH, NADH, insulin, and glutathione reductase (yeast, type III) were purchased from Sigma Chemical Company.

Tissue extract. Male Wistar rats weighing 150–200 g were used. nine ml of 0·9 % w/v NaCl were added per g of each tissue and the mixture was homogenized for 1 min in a Waring Blendor at 0–4°, then centrifuged at 10,000 g for 10 min. The supernatant obtained was employed as crude enzyme preparation.

Enzyme assay. (A) Fluorometric assay: The reduction of disulfide-type thiamine derivatives was determined by measuring the formed thiazole-type thiamine. The complete reaction mixture of the standard assay system contained 0·01 μmole of BTPD, 0·02 μmole of GSH, and aliquot enzyme preparation in a total volume of 2 ml of 0·2 M phosphate buffer, pH 7·4.* The reaction mixture was incubated at 30° for 5 min, then formed thiazole-type thiamine was determined by the thiochrome method.[16] One unit of enzyme activity was expressed as the formation of 1 μmole of thiazole-type thiamine per min corrected for nonenzymatic reaction under the above condition. Specific activity was the number of units per mg of protein.

(B) Spectrophotometric assay: The reaction catalyzed by the enzyme was coupled with that catalyzed by glutathione reductase, and the rate of oxidized glutathione formation from GSH and disulfide compounds was measured by following the decrease in absorbance of NADPH at 340 mμ in a Shimadzu SV-50A Spectrophotometer at 18°. The reaction mixture contained 200 μmoles potassium phosphate (pH 7·6), 1 μmole GSH, 0·2 μmole NADPH, 0·5 unit glutathione reductase, 12·5 μg partially purified enzyme, and 0·1 μmole substrate in a total volume of 2 ml. Enzyme activity was expressed as decrease of E_{340} per min corrected for any nonenzymatic reaction.

Protein estimation. Protein concentration was determined by the method of Lowry.[7]

* Substrates were not present at saturation levels because the nonenzymatic reduction was extremely fast so that at saturating concentrations it was difficult to assay the enzyme activity accurately. However, the reaction rate was linear with incubation time and protein concentration under the conditions used.

Determination of GSH. GSH was assayed by the fluorometric method of Cohn.[18]

Estimation of partition coefficient of thiamine derivatives. Partition coefficient of disulfide-type thiamine derivatives between benzene and 0·1 M phosphate buffer, pH 6·6, at 25° was estimated as previously reported.[19]

RESULTS

Enzymatic reduction of BTPD

As previously reported,[10, 20, 21] disulfide-type thiamine derivatives are slowly reduced nonenzymatically by GSH. The formation of thiazole-type thiamine was increased by the addition of liver extract, while the heat denaturated extract (100°, 3 min) had no such effect. (See Fig. 2.) The total amount of thiazole-type thiamine

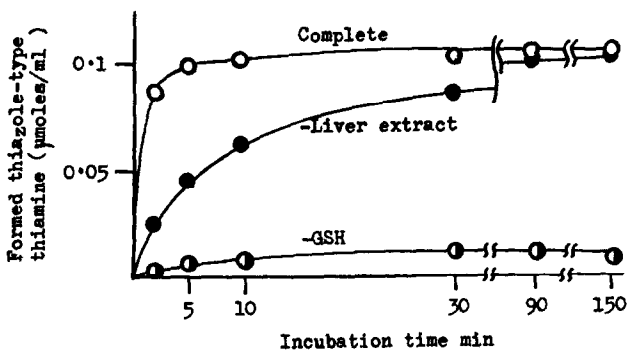

FIG. 2. Time course of enzymatic and nonenzymatic reduction of BTPD. The complete system contained, per ml of solution, 0·1 μmoles of GSH, 0·12 μmoles BTPD and liver extract (105 μg protein) in 0·1 M phosphate buffer, pH 7·4. The mixture was incubated at 37°.

TABLE 1. DISTRIBUTION OF ENZYME ACTIVITY IN RAT TISSUES

Tissue	Specific activity*
	$\times 10^{-3}$
Brain	4·0
Spleen	3·5
Kidney	6·5
Heart	4·4
Gut wall	4·3
Liver	33·1
Blood	0·7

* See "Materials and Methods".

formed was the same as that observed in the absence of the liver extract and was equivalent to the amount of GSH added. Table 1 shows the distribution of the enzyme activity in homogenates of several rat tissues. The highest specific activity was observed in liver, and little activity was found in blood.

Purification of enzyme

The enzyme was partially purified from rat liver as described below. All procedures were conducted at 0–4°.

Step 1. Crude extract: 10 g of acetone powder from rat liver was extracted with 130 ml of 0·05 M Tris buffer (pH 6·7) and centrifuged at 10,000 *g* for 10 min. The supernatant solution was used as the enzyme source.

Step 2. Acetone fractionation: 90 ml of chilled acetone were stirred into 125 ml of crude extract which was placed in a bath at −15°. After stirring for 3 min, the mixture was centrifuged. To the supernatant fraction, an additional 10 ml of chilled acetone was added and centrifuged in the same manner. The precipitate obtained was dissolved in 18 ml of 0·05 M Tris buffer (pH 6·7).

Step 3. DEAE-Sephadex chromatography: The solution obtained from step 2 was placed on a DEAE-Sephadex A-50 column (3·5 × 50 cm) equilibrated with 0·05 M Tris buffer (pH 6·7) and elution was accomplished with a linear gradient of NaCl (0–1 M) in the same buffer. The active fraction was pooled and dialyzed.

Step 4. CM-Sephadex chromatography: Dialyzed active fraction from step 3 was lyophilized, dissolved in 0·01 M sodium phosphate buffer (pH 5·8) and adsorbed on to a column (3·0 × 65 cm) of CM-Sephadex C-50 equilibrated with the same buffer. A linear gradient elution was carried out with 500 ml each of 0·01 M sodium phosphate buffer (pH 5·8) which contained no NaCl in the mixing vessel and 1 M NaCl in the reservoir. The active fraction was pooled, dialyzed, and lyophilized.

Step 5. Sephadex G-200 chromatography: A portion of lyophilized protein from step 4 was dissolved in 0·05 M phosphate buffer (pH 7·4) and layered on the G-200 column (1·2 × 80 cm) equilibrated with the same buffer and eluted with the same buffer. One ml fractions were collected and almost constant specific activity was observed between fractions 43 to 51. From the elution pattern of the G-200 column, the eluate from Step 5 seemed to be a single protein. However, disc electrophoresis on acrylamide gel revealed that the eluate contained some impure protein. Because of its extreme instability, further purification of the enzyme was unsuccessful, and active fraction from step 5 was pooled and used for the following study as a partially purified enzyme. Results of the purification are summarized in Table 2.

TABLE 2. PURIFICATION OF ENZYME

Step	Total protein (mg)	Total activity† (unit)	Specific activity†
1 Crude extract	4200	145	0.035
2 Acetone fractionation	268	35·5	0·13
3 DEAE-Sephadex	27	8·6	0·32
4 CM-Sephadex	6·7	4·8	0·71
5 Sephadex G-200*	0·072	0·08	1·11

* Sample from fraction 47.
† See the text.

Stoichiometry

GSH (1 × 10^{-4}M/L) was reacted under nitrogen with BTPD (1·5 × 10^{-4}M/l.) at 30°, pH 7·4, in the presence and absence of partially purified enzyme (0·5 μg protein/ml of reaction mixture) and the amount of thiazole-type thiamine and residual GSH were determined. The results shown in Fig. 3 suggested that BTPD and GSH reacted in equimolar amounts to form equimolar amounts of products. The reaction products

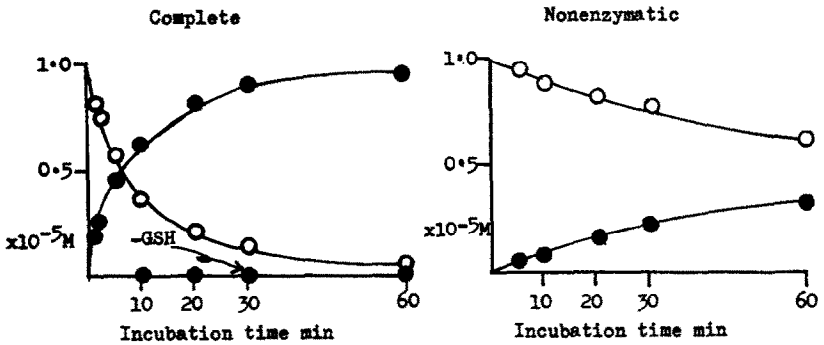

FIG. 3. Stoichiometry of reaction. ●—● Formed thiazole-type thiamine; ○—○ residual reduced glutathione. Procedure: see text.

were separated from the reaction mixture by thin layer chromatography (Keisel G, 1 N HCl). Two reaction products were identified, one of which reacted with Dragendorff reagent[22] and the other with ninhydrin reagent, and these had the same R_f values as *O*-benzoyl thiamine and S-propylmercapto glutathione, respectively.* From these results, this enzyme was proved to catalyze the transfer of hydrogen from GSH to disulfide derivatives.

$$B_1SSR\dagger + GSH \longrightarrow B_1SH + GSSR \quad (1)$$

TABLE 3. SPECIFICITY FOR HYDROGEN DONOR

Hydrogen donor	Formed thiazole-type thiamine μg	
	Nonenzymatic	Enzymatic
(A) GSH	0·76	4·69
Cysteine	0·73	0·19
Homocysteine	0·43	0·07
Cysteamine	1·03	0·13
Mercaptoethanol	0·35	0·05
Thiophenol	3·75	0·20
Thioglycolic acid	0·08	0·01
Bovin serum albumin	0·28	0·02
(B) GSH	0·18	1·74
Lipoic acid (reduced)	0·12	0·02
Ascorbic acid	0·02	0·01
(C) GSH	0·16	2·47
NADPH	0·03	0·04
NADH	0·03	0·05

Reaction mixture in a total volume of 2 ml was incubated for 5 min at 30°, pH 7·4, then formed thiazole-type thiamine was determined. Composition of reaction mixture excluding enzyme is shown below.
(A) 0·02 μmole of BTPD and 0·08 μmole of thiol.
(B) 0·01 μmole of BTPD and 0·02 μmole of hydrogen donor.
(C) 0·01 μmole of BTPD and 0·2 μmole of coenzyme. When GSH was used as the hydrogen donor, 0·02 μmole of GSH were used.

* The authentic sample of *O*-benzoyl thiamine was the product of Tanabe Seiyaku Co., Ltd. and S-propylmercapto glutathione was prepared as previously described.[11]
† Abbreviation: B_1SSR, disulfide-type thiamine derivatives; B_1SH, thiazole-type thiamine.

Specificity of hydrogen donor and acceptor

Since little thiazole-type thiamine was formed without GSH, this enzyme seemed to need GSH as hydrogen donor. From the results summarized in Table 3, this enzyme preparation was active only with GSH and a little activity was found with cysteine, cysteamine, or thiophenol under experimental condition. Neither NADPH nor NADH exhibited cofactor activity. Enzyme activity was determined with several disulfide thiamine derivatives and correlation between enzyme activity and partition coefficient of the derivatives is shown in Fig. 4. Generally, hydrophobic derivatives were more active than hydrophilic ones as substrate for the enzyme. In another series of disulfide derivatives of thiamine, symmetrical derivatives such as *O,O'*-dibenzoyl thiamine disulfide and *O,O'*-dibutyryl thiamine disulfide were shown to be a substrate for the enzyme, but the nonacylated derivative, thiamine disulfide, was a poor substrate for the enzyme (unpublished data).

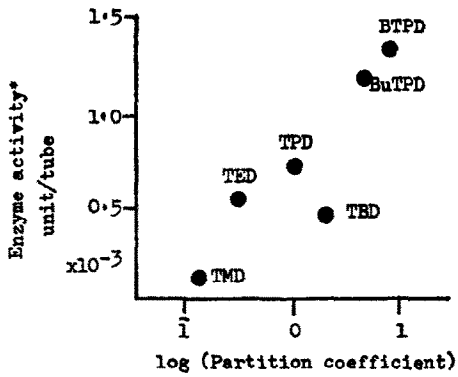

FIG. 4. Enzyme activity for disulfide-type thiamine derivatives. * Partially purified enzyme was used and standard assay was carried out (see text).

DISCUSSION

This investigation suggests that a transhydrogenase-type enzyme may play an important role in the reduction of disulfide-type thiamine derivatives to thiazole-type thiamine in animal tissues. In this enzymatic reaction, NADPH and NADH do not play the role of hydrogen donor as seen in Table 3 and this enzyme appears to be different from cystine reductase (EC 1.6.4.1), glutathione reductase (EC 1.6.4.2), lipoic dehydrogenase (EC 1.6.4.3), and protein disulfide reductase (EC 1.6.4.4). Other enzymes which catalyze GSH–disulfide exchange reaction have been reported.[23–30]

To clarify the identity of the enzyme, substrate specificity of the partially purified enzyme was assayed and results are shown in Table 4. This enzyme was active only with the disulfide-type thiamine derivative, and homocystine, cystine, cystamine, and insulin were poor substrate for this enzyme, so this enzyme seemed to be different from enzymes reported by Racker, Tomizawa, Katzen, Wendell and Nagai. Though there is no evidence that this enzyme does not catalyze the GSH-CoASSG exchange reaction reported by Chang *et al.*, this enzyme and GSH-CoASSG transhydrogenase are probably different. GSH-CoASSG transhydrogenase has been reported to have a

TABLE 4. SPECIFICITY FOR HYDROGEN ACCEPTOR

Substrate	E_{340}/min		
	Total	Nonenzymatic	Enzyme activity
TPhD	0·303	0·211	0·092
Homocystine	0·001	0·002	−0·001
Cystine	0·005	0·006	−0·001
Cystamine	0·002	0·002	0
Insulin	0·009	0·009	0

Procedure: see the text.

molecular weight of 12,300 and shows the highest specific activity in rat brain, whereas the present authors' enzyme has a molecular weight of about 50,000 from gel-filtration studies, and shows a higher activity in liver than in brain. Thus, the enzyme described in this paper seems to be different from other GSH–disulfide transhydrogenases described in the literature. The enzyme differs from glutathione dehydrogenase (EC 1.8.5.1), which has not been found in rat liver (unpublished data). No other substrate but the synthetic disulfide-type thiamine derivatives are known for this enzyme, and so little information is available concerning its physiological role at present.

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