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THE IDENTITY OF ENZYMES REDUCING A THIAMINE DISULFIDE DERIVATIVE AND CYSTINE DERIVATIVES VIA THIOL-DISULFIDE EXCHANGE

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Abstract—In earlier work*, we have studied a labile enzyme activity catalyzing an exchange between thiol and acceptor. Glutathione (GSH) was used as the thiol and a number of low molecular weight substances such as cystine and GSH-disulfide derivatives, *S*-sulfocysteine ($CySSO_3H$), *S*-sulfoglutathione and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) were used as acceptor substrates in the thiol transfer reaction. This broad substrate specificity led us to the tentative suggestion that thiamine disulfide derivatives also were acceptor substrates to the thioltransferase† activity, which is confirmed in this study. The methods used for the resolution of enzymes and substrate specificity were: (1) isoelectric focusing, (2) CM-cellulose chromatography, (3) labelling of the thioltransferase with [^{35}S]GSH, (4) gel filtration on Bio-Gel P-150, and (5) investigation of ratios of the specific activities of GSH-linked enzymes in different tissues. Generally it was found that bovine tissue had higher specific thioltransferase activity than rat tissue. GSH *S*-aryltransferase (EC 2.5.1.13) had quite different activity ratios from those obtained with the enzyme involved in cystine and thiamine disulfide reduction. This result, and dissimilar chromatographic behavior, indicate that GSH *S*-aryltransferase is not involved in disulfide reduction.

The occurrence of thiamine disulfide derivatives was first reported by Myrbäck *et al.* [1]. They also suggested that a thiol, GSH, might take part in thiamine disulfide reduction. Their results were challenged however by *e.g.* Schönberg and Sperber [2]. Later Suomalainen *et al.* confirmed the appearance of diphosphothiamine disulfide in aerobically cultivated baker's yeast [3]. Several thiamine disulfides have been prepared and tested in biological systems [4–8]. Some of these disulfides, such as thiamine propyldisulfide, were more effective than thiamine in promoting growth (in the *in vivo* formation of thiaminepyrophosphate) and in absorption from the intestine [5–9]. It was confirmed that a reducing agent is required for conversion of thiamine disulfide and thiamine alkyldisulfides to thiamine [7, 8]. Kohno *et al.* have shown that the thiol–disulfide exchange between glutathione and thiamine disulfide derivatives is enzyme-catalyzed in rat liver [10]. The TT-BTPD was reported to be identical with neither protein disulfide reductase (NADPH) (EC 1.6.4.4) nor enzymes catalyzing GSH-disulfide exchange. This TT-BTPD preparation does not exclude the presence of microsomal enzymes catalyzing thiol–disulfide interchange as in the case of glutathione:insulin transhydrogenase [11, 12].

We have recently characterized the thiol–acceptor exchange concerning GSH and cystine derivatives to involve thiol–transfer rather than hydrogen–transfer [13]. The ring opening–closing reactions of 4-methylthiazolium salts, a prerequisite for the formation of disulfides of thiamine, have been examined and rate constants estimated [14].

Further studies on thiamine disulfides revealed that the TT-BTPD activity profile almost coincided with that of GSH *S*-aryltransferase [15]. Our own results concerning the resolution of GSH-linked enzymes in rat liver were similar [16]. The thioltransferase activities with cystine derivatives and 5,5′-dithiobis(2-nitrobenzoate) (DTNB) as disulfide substrates had some activity peaks coinciding with those of GSH *S*-aryltransferase. We have used CM-cellulose chromatography, isoelectric focusing, gel filtration and response to treatment with GSH, [^{35}S] GSH and dithioerythritol (DTE) in order to resolve the GSH-linked enzyme activities involved. The major emphasis of this

* See refs. 13, 16, 20, 22, 28.

† Thioltransferase was introduced as a name for enzymes earlier described as transhydrogenases, glutathione:cystine oxidoreductase (EC 1.8.4.1-1.8.4.4) [13, 22].

Abbreviations: BTPD, *O*-benzoyl thiamine propyl disulfide; CoASSG, CoA-GSH mixed disulfide; CySSG, CySH-GSH mixed disulfide; $CySSO_3H$, *S*-sulfocysteine; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); LDH, lactate dehydrogenase; AT, GSH *S*-aryltransferase; GR, glutathione reductase; TT-BTPD, GSH-thiamine disulfide derivative thioltransferase; TT-$CySSO_3H$, GSH *S*-sulfocysteine thioltransferase; TT-DTNB, GSH-DTNB thioltransferase.

investigation is directed towards the enzymes active with a thiamine disulfide derivative as substrate and the role of thioltransferase and GSH *S*-aryltransferase in disulfide reduction.

MATERIALS AND METHODS

Materials. *O*-Benzoyl thiamine propyl disulfide (BTPD) was kindly supplied by Dr. Kohno, Products Res. Dev. Lab., Tanabe Seiyaku Co., Ltd., Osaka, Japan. *S*-Sulfocysteine ($CySSO_3H$) was synthesized according to Segel and Johnson [17], the mixed disulfide of CoA and glutathione (CoASSG) according to Eriksson by a modified procedure [18], and the mixed disulfide of cysteine and glutathione, (CySSG) according to Eriksson and Eriksson [19]. DTE, GSH, GSSG, NADPH and glutathione reductase, NAD(P)H:glutathione disulfide oxidoreductase (EC 1.6.4.2) (yeast) were obtained from Sigma; 5,5'-dithiobis(2-nitrobenzoic acid) from Aldrich; 3,4-dichloronitrobenzene from Schuchardt; Ampholine carrier ampholytes from LKB; Sephadex G-25 Fine from Pharmacia Fine Chemicals; CM-cellulose from Whatman; Bio-Gel P-150 400 mesh from Bio-Rad; Diaflo membrane filter XM50 from Amicon; Aquasol® from NEN and [^{35}S] GSH (sp. act. 5 Ci/mole) from Schwarz/Mann.

Separation of rat liver enzymes. Livers from Sprague–Dawley male rats were homogenized in 4 volumes 0·25 M sucrose, 1 mM EDTA. The homogenate was centrifuged at 145,000 *g* for 75 min. The supernatant was chromatographed on a Sephadex G-25 Fine bed, the gel volume was 5-fold the volume of the sample. At subsequent isoelectric focusing or CM-cellulose chromatography the gel was equilibrated with water or 10 mM sodium phosphate pH 6·2, 1 mM EDTA, respectively.

Isoelectric focusing was carried out on a 6 ml sample (containing 100 mg protein) from the effluent of the Sephadex-column. The pH gradient of 1% Ampholine No. 8141 in a 0–50% sucrose gradient was established during isoelectric focusing for 43 hr at 4° and 300V.

In order to resolve TT-BTPD and thioltransferase during CM-cellulose chromatography, we compared their elution patterns upon treatment with GSH [20].

Rat liver homogenate from 44 g tissue was used. The postmicrosomal supernatant was adjusted to pH 8; one part served as control, the remaining two thirds were incubated with 2 mM GSH for 10 min, whereupon one half of this volume was treated with 5 mM DTE for 30 min. The samples were each chromatographed on separate Sephadex and CM-cellulose columns. The protein effluents from the Sephadex G-25 steps were applied on CM-cellulose columns, and unadsorbed material was washed off with the buffer used for equilibration. A linear concentration gradient, 0–0·2 M NaCl, containing 10–50 mM sodium phosphate (pH 6·2) and 1 mM EDTA, 500 ml in total, was used to elute the adsorbed enzyme activities in 7–10 ml fractions.

Enzymatic assays. GSH *S*-aryltransferase was assayed spectrophotometrically by measuring at 344 nm the formation of *S*-2-chloro-4-nitrophenyl-glutathione from 3,4-dichloronitrobenzene and GSH as substrates [21]. The determinations were made at 30° using an extinction coefficient of 10 mM^{-1} cm^{-1}. The formation of 1 μmole product per min was defined as a unit of enzymatic activity. The reaction system contained 1 mM 3,4-dichloronitrobenzene (added as a 20 mM solution in ethanol), 5 mM GSH, 0·17 M Tris buffer (pH 8·0), 1 mM EDTA and enzyme in a final volume of 1 ml.

Glutathione reductase was assayed spectrophotometrically at 340 nm by recording the oxidation of NADPH. The reaction system consisted of 1 mM GSSG, 0·1 mM NADPH, 0·17 M sodium phosphate (pH 7·6), 1 mM EDTA and enzyme in a total volume of 1 ml.

Thioltransferase, active with GSH and cystine derivatives, was assayed by coupling to glutathione reductase. *S*-Sulfocysteine and *S*-sulfoglutathione were shown to be substrates to the same enzyme activity, equations 1–3 [22].

$$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^- \quad (1)$$

$$CySSG + GSH \rightleftharpoons GSSG + CySH \quad (2)$$

$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ \quad (3)$$

The assay system consisted of 0·5 mM GSH, 2·5 mM $CySSO_3H$, 0·1 mM NADPH, 0·4 unit of yeast glutathione reductase, 0·145 M sodium phosphate (pH 7·6), 1 mM EDTA and enzyme (50–100 μl) in a total volume of 1 ml.

Thioltransferase active with BTPD as substrate was assayed as the thioltransferase activity described above with the substitution of 0·1 mM BTPD (added as a 2 mM solution in ethanol) for 2·5 mM $CySSO_3H$. The use of one of the following substrate concentrations: 0·15 mM CoASSG, 0·25 mM CySSG, 0·5 mM $GSSO_3H$, 2·5 mM $CySSO_3H$ and 0·1 mM BTPD in the assay of thioltransferase in rat liver homogenate resulted in a similar magnitude of activity ranging between 1·15 and 1·60 μmoles/min g under the assay conditions used.

Thioltransferase activity visualized with DTNB as disulfide substrate was based on the formation of 3-carboxy-4-nitrobenzenethiolate measured at 412 nm in a Beckman DB-G spectrophotometer. The reaction system contained 50 μM GSH, 50 μM DTNB, 0·17 M sodium phosphate (pH 6·0), 1 mM EDTA and 50–100 μl enzyme.

The reaction velocities of thioltransferase activities were always corrected for the contribution of the nonenzymatic reaction between the thiol and the acceptor. Protein concentration was estimated according to the method described by Kalckar [23].

Radioactivity assay. The effluent of the CM-cellulose column was analyzed for the presence of incorporated [^{35}S]GSH in fractions catalyzing thiol–disulfide exchange. Furthermore the treatment with [^{35}S]GSH

aimed to show the transfer of free transhydrogenase to a more acidic enzyme–GSH mixed disulfide. The second peak of untreated thioltransferase, devoid of bound GSH, obtained from the CM-cellulose column, was incubated for 10 min at pH 8·0 with 120 μM [^{35}S] GSH (sp. act. 5 Ci/mole) [20]. The buffer was changed to the one used for CM-cellulose chromatography by passage through Sephadex G-25 equilibrated with the 10 mM sodium phosphate (pH 6·2) containing 1 mM EDTA. Radioactivity was determined in 1 ml aliquots of effluent from the CM-cellulose column, added to 10 ml Aquasol® and counted in a Beckman LS-100 scintillation counter.

RESULTS AND DISCUSSION

Isoelectric focusing of rat liver supernatant. Isoelectric focusing was used in order to investigate whether there are components of acidic nature catalyzing thiol–disulfide exchange and consequently not bound to the ion exchanger and thus eliminated before the analysis on CM-cellulose. The distribution pattern of the activities in question is seen in Fig. 1, where the most acidic component of the activity profile of TT-BTPD partly coincides with the peak of glutathione reductase and the other activity components also have isoelectric points that should permit them to bind to the CM-cellulose ion exchanger. The TT-BTPD peak coinciding with that of glutathione reductase is explained by the nonsaturated conditions of glutathione reductase in the test system. The remaining TT-BTPD activity coincides mainly with the thioltransferase activity. There is, however, a region between the peaks of glutathione reductase and thioltransferase in Fig. 1, where

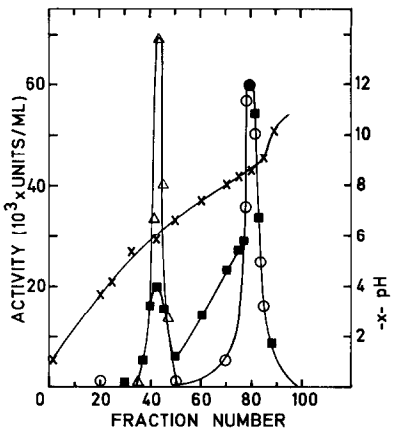

Fig. 1. Isoelectric focusing of a rat liver supernatant after passage through Sephadex G-25. The effluent, 6 ml containing 100 mg protein, was introduced into the middle of the 100 ml column. The pH-gradient of 1% Ampholine No. 8141 in a 0–50% sucrose gradient was established during isoelectric focusing for 43 hr at 4° and 300V. Fractions of 1–1·5 ml were collected. TT-BTPD, —■—; TT-CySSO$_3$H, —○—; GR activity × 1:10, —△—.

TT-BTPD does not fully coincide with the activity profile of thioltransferase.

Thioltransferase is the only GSH-linked enzyme studied by us to specifically change its elution profile from CM-cellulose after treatment with GSH [20, 22]. (See Materials and Methods). It was consequently investigated whether TT-BTPD showed the same specific sensitivity and thus be assignable to thioltransferase.

The response in elution pattern from the CM-cellulose to the treatment with GSH and DTE is observed in Fig. 2. The activities of TT-BTPD and thioltransferase coincide in Fig. 2a–c. GSH *S*-aryltransferase has one of its components coinciding with the basic activity component catalyzing thiol–disulfide exchange in Fig. 2a and b. The GSH content of the GSH-treated portion, without DTE, obtained according to the procedure described in Materials and Methods, was lowered during the Sephadex-chromatography because 7 volumes of gel was used instead of 5 volumes. The reason for the change in the Sephadex volume was to investigate whether the activity profile of thioltransferase

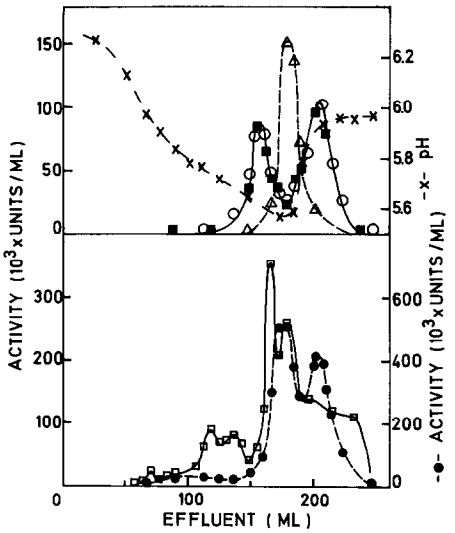

Fig. 2a

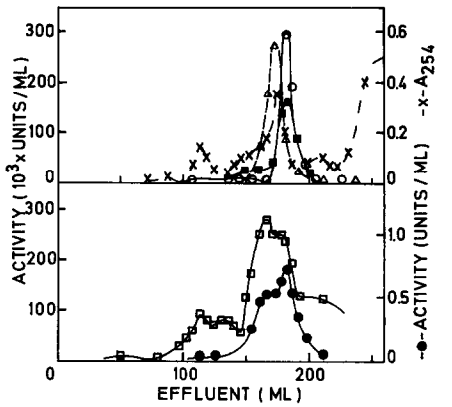

Fig. 2b

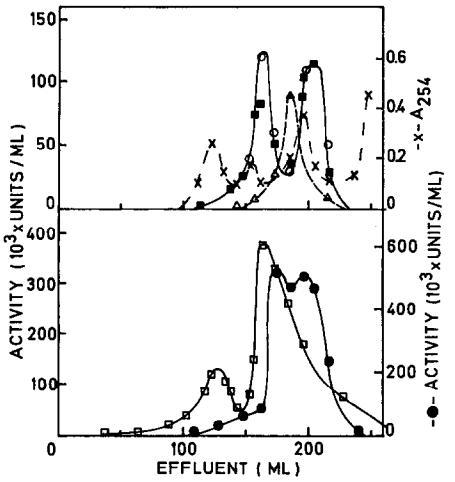

Fig. 2c

Fig. 2. CM-cellulose chromatography of rat liver supernatant from 44 g tissue. The supernatant was divided into three parts for treatment and subsequent chromatography on separate Sephadex G-25 and CM-cellulose (2 × 12 cm) columns according to the procedure described in Materials and Methods. (a) One third of the supernatant was used as a non-treated control of the shape of the elution profile of the enzymes. The sample was adjusted to pH 8·0 before chromatography. TT-DTNB, —□—; AT, —●—; GR activity × 1:10. The other symbols are given in Fig. 1. (b) Treatment of the remaining supernatant with 2 mM GSH at pH 8·0 transferred all thioltransferase activity into the acidic peak separated from GSH *S*-aryltransferase [20]. This acidic sample was totally transferred to a basic component by incubation of half of the volume with 5 mM DTE. GR activity × 1:5. The symbols are given in Figs. 1 and 2a. (c) A similar transfer of acidic thioltransferase to a basic component was achieved by chromatography on excess Sephadex gel (7 volumes instead of 5 volumes of the sample volume), which indicated an equilibrium between GSH and enzyme. GR activity × 1:20. The symbols are given in Figs. 1 and 2a.

could be still better resolved from the one of GSH *S*-aryltransferase.

As is seen in Fig. 2c the activity profiles of thioltransferase and TT-BTPD were each easily divided into two peaks, from one original acidic peak, after the GSH-incubation (cf. Fig. 2, ref. 20). A partial separation between GSH *S*-aryltransferase and thioltransferase was observed, which was not obtained after the control CM-cellulose chromatography (Fig. 2a). The separation of GSH *S*-aryltransferase and thioltransferase is however, still better resolved after isoelectric focusing [16, 22].

To gain further support that GSH was bound to TT-BTPD, [^{35}S] GSH labelling was used to give more direct evidence.

It is seen in Fig. 3 that a conversion of the basic form of thioltransferase into the acidic one has occurred, coinciding with a peak of radioactive GSH. It is noteworthy that the activity ratio between TT-BTPD and thioltransferase is different in the two peaks, a matter which is discussed below. In addition, the thioltransferase activity with DTNB as disulfide substrate has three peaks, two of which coincide with the TT-BTPD and thioltransferase activity profiles. The assay system with DTNB has the advantage that thioltransferase activity is directly measured without coupling to glutathione reductase as in the common test system. Furthermore, if TT-BTPD would obtain a different elution profile compared with thioltransferase, we expected that this deviating activity profile also could be followed with the less specific test system containing DTNB. So far, any distinct TT-BTPD peak separated from the thioltransferase activity has not been obtained.

The activity ratios between different purification steps and substrates were investigated; a difference in ratios would be indicative that there were different enzymes for the TT-BTPD and thioltransferase reactions.

The activity with BTPD as substrate was 1·528 μmoles/min and g fresh liver in the postmicrosomal fraction. After Sephadex G-25 and CM-cellulose chromatography and ultrafiltration on Diaflo membrane XM50, 0·327 μmole/min g fresh liver remained,

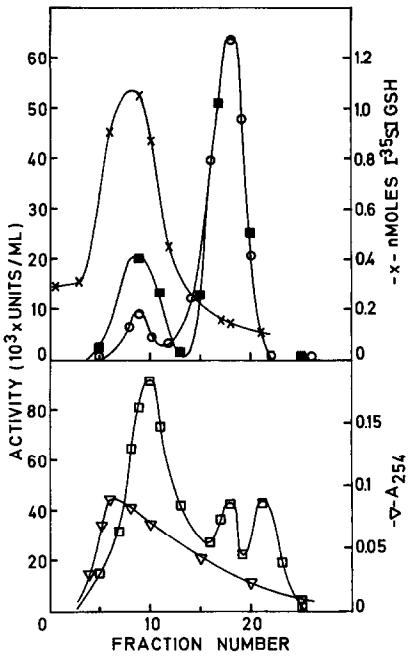

Fig. 3. CM-cellulose chromatography of thioltransferase, labelled with GSH. The labelling of the GSH-depleted thioltransferase is described in Materials and Methods. The column chromatography is described in Materials and Methods and Fig. 2 with the following alterations: the column size was 2 × 4 cm and the linear concentration gradient was 400 ml. Fractions of 5 ml were collected. The symbols are given in Figs. 1 and 2.

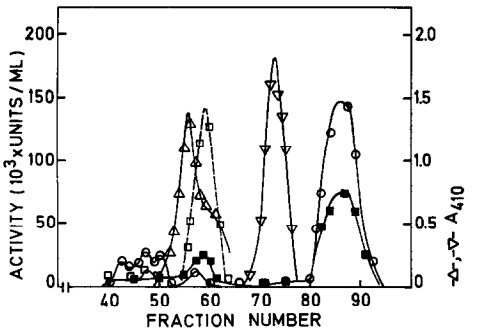

Fig. 4. Gel filtration on Bio-Gel P-150 400 mesh. A thioltransferase fraction obtained after CM-cellulose chromatography was concentrated by ultradialysis and 1·5 ml sample with the activity of 10 μmoles/min per ml was introduced onto the 2 × 35 cm P-150 column. The column was equilibrated with 50 mM Tris buffer (pH 7·5), 1 mM EDTA and 0·1 M KCl. Fraction No. 88 had a sp. act. of 1·4 μmoles/min and mg protein tested with $CySSO_3H$ as substrate. Fractions of 1 ml were collected. TT-DTNB activity × 1:10. Hemoglobin, —△—; cytochrome *c*, —▽—. The other symbols are given in Figs. 1 and 2.

corresponding to a 21·4 per cent yield. The corresponding values for $CySSO_3H$ as substrate were 1·145 and 0·243 μmoles/min and g liver. The resulting yield of 21·2 per cent indicates that the recovery for the two activities was the same and thus the enzyme activity can be ascribed to one and the same liver component.

The separation techniques described so far utilize the ionic character of the sample. Adsorption chromatography of GSH *S*-aryltransferase and TT-BTPD was studied by Noda [15], but this method did not separate the two activities.

We investigated the two combined CM-cellulose components by gel filtration. We and others have observed that the thioltransferase activities are easily inactivated especially during Sephadex G-100 chromatography [refs. 10, 24–26 and unpublished results]. Bio-Gel P-150 was used as gel filtration medium. Under the assay conditions thioltransferase, which has a molecular weight of 50,000 daltons, obtained from Sephadex G-50 chromatography [27], dissociates or retards to a molecular weight corresponding to 3000–5000 daltons (Fig. 4). Some reference proteins were included in the sample to enable us to estimate the molecular weights of the active components as is seen in Fig. 4. Less than one tenth of the eluted TT-BTPD activity was found in a fraction catalyzing GSH–DTNB exchange and the main part of the TT-BTPD was recovered in the thioltransferase peak. Association and dissociation phenomena might explain the drastic change seen in the molecular weight distribution pattern. Evidence is provided by the presence of substantial thioltransferase activity around Fraction No. 45 where LDH was eluted, which defines the exclusion limit of the column. LDH, however, was only found in Fractions 43–47. Enzymatic degradation of the thioltransferase cannot be ruled out because the separation on P-150 lasts about 5 days. The low molecular weight thioltransferase peak does not contain thiols or GSSG which would give an additional activity. This active fraction is free from turbidity and contains 0·1 mg protein per ml in the peak fraction.

We have suggested earlier [27] that a change in the substrate specificity occurred in some separation steps as during DEAE-cellulose chromatography. Furthermore, prolonged application time of the sample onto the CM-cellulose gel resulted in acidic degradation products which was confirmed by a poor yield and by isoelectric focusing [16]. Additional separation steps lead to a complete loss of enzyme activity (cf. ref. 10) with cystine derivatives as disulfide substrates, but thiol–disulfide exchange was still furnished between GSH and DTNB by the enzyme [27]. After CM-cellulose separation the total activities with DTNB and $CySSO_3H$ as disulfide substrates, as is seen in Fig. 2, were in a ratio of 4:1. The Bio-Gel P-150-separation illustrated in Fig. 4 indicated that a much higher ratio was obtained, thus supporting a change in substrate specificity.

The low TT-BTPD coinciding with glutathione reductase in Fig. 1 is interpreted [28] as a catalytic contribution to the thiol–disulfide exchange by rat liver glutathione reductase. The same stimulation by increasing the yeast glutathione reductase could not be obtained. The region between the glutathione reductase and the thioltransferase in Fig. 1, where an irregular profile of TT-BTPD is present, contains GSH *S*-aryltransferase and DTNB-thioltransferase activities which contribute to a nonspecfic GSH–thiamine disulfide interchange [22]. It should also be noted that an isoelectric focusing experiment lasts for about 40 hr during which time alterations of the enzyme molecule could occur. GSH *S*-aryltransferase and $CySSO_3H$-thioltransferase each contribute similarly to DTNB-thioltransferase activities which was investigated earlier [16, 22].

The reason for different ratios between TT-BTPD and thioltransferase in Fig. 3, in contrast to the results in Fig. 2a and c, is interpreted as ageing and consequent change in substrate specificity, an explanation that is supported by the results obtained in Fig. 2b.

We investigated the distribution of GSH *S*-aryltransferase, thioltransferase and TT-BTPD in rat and bovine tissues in order to find out if there exists a close relationship between any of the enzymes measured. It is obvious from the distribution pattern in Table 1 that there is a closer correspondence between thioltransferase and TT-BTPD activities than between GSH *S*-aryltransferase and TT-BTPD activities, the matter is most pronounced in rat liver and bovine tissues. Further evidence for the existence of one single enzyme activity catalyzing thiol–disulfide exchange with thiamine disulfide derivatives as well as cystine derivatives emerges from the regular ratio of the specific enzymatic activity, with the two disulfide substrates.

Table 1. Specific activities of GSH-linked enzymes of a number of bovine and rat tissues*

Tissue	Enzyme activity†	Specific activity (nmoles formed product/min . mg protein)									
		Brain	Heart	Intestine	Kidney	Liver	Lung	Pancreas	Spleen	Suprarenal gland	Thymus
Supernatant	Protein mg/ml	3·40	5·1	24·2	16·9	36·9	8·10	6·30	5·30		
Rat	TT-BTPD	5·30	1·57	7·35	2·42	6·18	3·70	1·59	17·75		
	TT-$CySSO_3H$	4·42	6·86	12·1	4·02	12·2	5·55	3·65	16·4		
	TT-DTNB	3·24	15·3	2·73	5·26	6·25	17·5		31·1		
	GR	21·0	5·00	96·0	107	60·0	22·0	13·0	25·0		
	AT	3·82	0·89	0·35	0·79	17·1	0·79	1·75	0·53		
Supernatant	Protein mg/ml	7·40	7·00	10·2	21·7	40·7	30·9	37·0	41·7	37·7	9·00
Bovine	TT-BTPD	46·0	22·6	21·2	15·0	9·05	9·65	8·20	8·96	6·30	31·6
	TT-$CySSO_3H$	64·2	34·4	29·1	19·9	11·5	11·8	11·9	11·6	8·0	56·5
	TT-DTNB	12·6	45·3	4·85	30·0	54·0	10·4	6·00	3·84	20·0	16·7
	GR	55·7	30·6	21·5	47·8	48·2	17·2	22·2	17·3	6·0	48·2
	AT	1·0	0·49	0·17	0·21	0·34	‡	‡	‡	0·12	0·25

* The specific activities are not adjusted for the contribution of activity by endogenous GSH, except for thioltransferase activity with DTNB as substrate. Between animals of the same species, a difference of 30% in specific activity was observed.

† Abbreviations used: TT-BTPD, GSH-thiamine disulfide derivative thioltransferase; TT-$CySSO_3H$, GSH *S*-sulfocysteine thioltransferase; TT-DTNB, GSH-DTNB thioltransferase; GR, glutathione reductase and AT, GSH *S*-aryltransferase.

‡ Values were less than 0·1 nmole/min . mg.

A reason why earlier investigators could not find coinciding activity profiles of the thioltransferase with thiamine disulfide derivatives and cystine derivatives as substrates is probably the kind of purification procedure chosen and the concomitant change in substrate specificity [10, 15]. By following the coinciding TT-BTPD and thioltransferase activity profiles in several purification steps, we conclude that the major activity catalyzing GSH-thiamine disulfide exchange is identical with thioltransferase and therefore suggest that the reaction pathway between GSH and thiamine disulfide derivatives proceeds via a thiol transfer. The enzyme activity in question is thus properly defined as a GSH:acceptor thioltransferase [13].

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