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Evidence for a Glutathione-dependent Interconversion of Two Forms of a Thioltransferase from Rat Liver Catalyzing Thiol-Disulfide Interchange

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In studies on rat liver two forms of a thioltransferase (EC 1.8.4.1), an enzyme catalyzing thiol-disulfide exchange, were separated by CM-cellulose chromatography and isoelectric focusing. Incubation with glutathione increased the activity of the more acidic form and caused a concomitant loss of the basic form. This effect could be reversed by dithioerythritol. Glutathione disulfide did not effect the conversion of one form of the enzyme into the other. Treatment of the basic component with [^{35}S]-GSH resulted in incorporation of radioactivity which was recovered in the acidic thioltransferase peak found upon repeating the chromatography on CM-cellulose. These experimental findings are consistent with a mechanism of interconversion involving thiol-disulfide interchange of GSH and a disulfide group of the enzyme.

In a previous paper we reported that two forms of a thioltransferase, an enzymatic activity in rat liver catalyzing thiol-disulfide interchange with glutathione (GSH) and the mixed disulfide of cysteine and glutathione (CySSG),* could be separated by CM-cellulose chromatography and isoelectric focusing.[1] These forms were distinct from similar activities observed in aged rat liver preparations. The ratios of the reaction velocities obtained with a variety of glutathione sulfenyl derivatives (such as CySSG, *S*-sulfoglutathione, and the mixed disulfide of coenzyme A and glutathione) as well as with other disulfides and thiosulfate esters were the same for both fractions of the CySSG-thioltransferase activity, indicating that these are different forms of the same enzyme.[2] The distribution of the total CySSG thioltransferase activity between its two different forms varied with experimental conditions in a way which suggested that the variations could be ascribed to dissociation or association phenomena. The present investigation was undertaken to further elucidate the mechanism of interconversion of the two forms of the enzyme.

MATERIALS AND METHODS

In addition to the materials and methods described in the previous paper,[1] dithioerythritol (DTE) (Sigma); Diaflo ultrafiltration membranes (Amicon); [^{35}S]-GSH (Schwarz/Mann), and Aquasol (NEN) have been used.

Pretreatment of homogenates from rat liver before CM-cellulose chromatography. Livers from male Sprague-Dawley rats (45 g) were homogenized shortly after removal in 4 volumes of 0.25 M sucrose and the resulting homogenate was centrifuged for 1 h at 145 000 *g*. The supernatant (134 ml) was adjusted to pH 8.0 and divided into three equal parts. One part served as a control in the subsequent CM-cellulose chromatography. The remaining two parts were incubated at pH 8.0 for 5 min with 2 mM GSH and one of these was afterwards treated with 1 mM dithioerythritol for another 5 min. The three preparations were then chromatographed on separate Sephadex G-25 columns of equal sizes to remove GSH and dithioerythritol and to change the solvent of the samples to the buffer used for equilibration of the CM-cellulose columns. The sample volume was 20 % of the Sephadex G-25 bed volume.

CM-cellulose chromatography. Fractionation of the three samples on CM-cellulose was carried out simultaneously under identical conditions, and the effluent was collected in 7 – 10

* Unusual abbreviations: CySSG, the mixed disulfide of cysteine and glutathione; $CySSO_3H$, *S*-sulfocysteine; DTE, dithioerythritol.

ml fractions. Three CM-cellulose columns (2 × 14 cm) were equilibrated with 10 mM sodium phosphate buffer – 1 mM EDTA (pH 6.2). Each sample was introduced into a separate column, which was then washed with the buffer used for equilibration until the UV absorption of the effluent vanished. Elution was carried out with a linear gradient formed from 250 ml 10 mM sodium phosphate – 1 mM EDTA (pH 6.2) in the mixing chamber and 250 ml 50 mM sodium phosphate – 1 mM EDTA 0.2 M NaCl (pH 6.2) in the reservoir.

[^{35}S]-GSH labelling of thioltransferase collected from CM-cellulose columns. Pooled fractions were concentrated two-fold on a PM 30 Diaflo membrane, adjusted to pH 8.0, and incubated for 10 min with 120 μM [^{35}S]-GSH (spec. act. 5 Ci/mol). The incubation mixture was desalted on Sephadex G-25 according to the procedure described above and subsequently rechromatographed on CM-cellulose to determine whether the basic form of the thioltransferase had incorporated [^{35}S]-GSH to give the acidic form. Radioactivity was determined by liquid scintillation counting of 1 ml samples in 10 ml Aquasol.

Ultrafiltration. The first and second peaks of the thioltransferase activity, obtained after CM-cellulose chromatography, were collected separately and each was subjected to a series of ultrafiltrations on membrane filters of increasing porosities. The collected material (37.5 ml) was first concentrated to a volume of 13.5 ml on a PM 30 membrane. This concentrate was then filtered on an XM 50 membrane until a volume of 4 ml remained in the cell. Finally, this remaining material was concentrated to 1 ml on an XM 100 membrane. Both peaks were treated in the same manner at 4 °C and the enzyme activities in both concentrates and filtrates were measured.

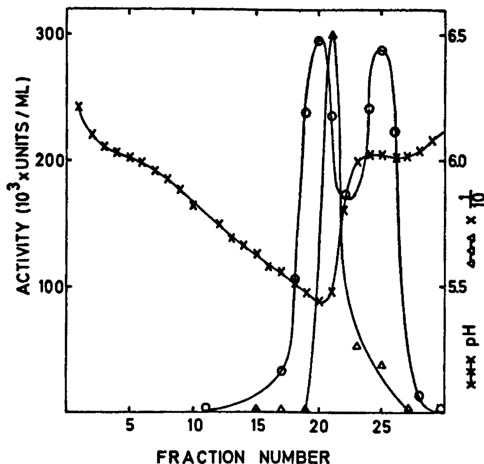

Fig. 1. CM-cellulose chromatography of the supernatant fraction from 30 g of rat liver after chromatography on Sephadex G-25. The sample was eluted with a linear salt gradient. The thioltransferase activity appears in two peaks of similar sizes if the sample volume is about 20 % of the volume of the Sephadex gel bed. (See Materials and methods). The enzymatic activities are: O, thioltransferase measured with $CySSO_3H$ and GSH as substrates; △, glutathione reductase.

RESULTS AND DISCUSSION

Fig. 1 demonstrates a typical elution profile obtained after chromatography of the supernatant fraction from rat liver on a CM-cellulose column. Gradient elution separated the thioltransferase activity into two components. The distribution of the activity was dependent on the experimental conditions and varied from a distribution in which the first peak contained the major part of the activity (*cf.* Ref. 1, Fig. 2C) to a distribution in which the second peak dominated. The possibility was considered that the appearance of two peaks was an artifact produced by the steep rise in the pH of the effluent which coincided with the appearance of thioltransferase activity (Fig. 1). The initial decrease in the pH of the effluent is caused by substitution of sodium ions of the gradient for hydrogen ions of the ion-exchange matrix and the subsequent rise in pH indicates the establishment of new ionic equilibria. However, glutathione reductase, which appears in the same region of the elution profile, is not split into two components. Furthermore, the two peaks of thioltransferase activity were also obtained when a gradient having less buffering capacity was used, conditions which delay the pH rise (*cf.* Ref. 1, Fig. 2C). Finally, isoelectric focusing also established the existence of two separable components of thioltransferase activity and thus eliminated the possibility that the appearance of the two peaks was an artifact due to a particular separation technique.

It was discovered that the size of the column beds had a marked effect on the distribution of the thioltransferase activity between the two peaks. An increase of the second component at the expense of the more acidic one was correlated with an increase in the column bed size using a constant sample volume. This effect was most clearly observed with changes in the column bed size for gel filtration on Sephadex

G-25, which preceded the CM-cellulose chromatography, but also to a smaller extent in the latter separation. It is well known that Sephadex can retard substances by adsorption due to hydrophobic interactions,[3,4] and the possibility was therefore considered that non-covalently bound lipids could be removed from the enzyme, thereby causing conversion of the more acidic thioltransferase component into the other form.

That lipids are indeed separated from the protein fraction of rat liver supernatant by gel filtration is suggested by the appearance of a very slowly migrating yellowish zone, which causes the fractions it is eluted in to appear turbid. In order to test the possibility that lipids constitute the difference between the two peaks of thioltransferase activity, the supernatant fraction from rat liver was treated, after gel filtration and before ion-exchange chromatography, with the lipid fraction obtained from the Sephadex column. However, no difference between the treated sample and the untreated control could be detected with respect to the distribution of the thioltransferase activity (22 and 78 % of the total activity in the first and second peaks, respectively, for both samples). Treatment of the sample with different mixtures of purified lipids known to be present in rat liver did not change the relative amounts of the two components either.

The influence of the column bed size on the distribution pattern of thioltransferase activity can be explained by assuming that the two components are in a dissociation-association equilibrium. The dissociation could involve removal of a low-molecular weight compound, as in the hypothesis discussed above, or consist of an oligomer-monomer transition by a protein containing subunits. In order to test the latter possibility the two forms of thioltransferase obtained after CM-cellulose chromatography were compared with respect to their molecular weights by means of ultrafiltration on Diaflo filters of different porosities. Ultrafiltration was chosen as the method for molecular weight determination instead of gel filtration, because the latter procedure causes inactivation of thioltransferase activity and is known to remove, for example, GSH from the thioltransferase. A preliminary estimation by gel filtration on Sephadex G-50 of the molecular weight of the basic form of the thioltransferase indicated a value of about 50 000; and the molecular weight of the acidic form was therefore expected to be a multiple of this value, if dissociation and association of subunits would be the mechanism of the interconversion of the two enzyme forms. The results of ultrafiltration of the two forms are shown in Table 1. Care was taken to use membranes with pore sizes such that full and partial retention of the enzyme activity in the ultrafiltration cell were both obtained. This is essential, because both oligomer and monomer may be too large to penetrate the membrane filter or both may be too small to be retained at all. The extent of retention and penetration of the two components was not significantly different on membrane filters of the same porosity, thus excluding major differences in molecular weights. The retention characteristics of the membranes used would have revealed differences between the two components if the molecular weights were 50 000 and a multiple thereof. Consequently, these results seem to

Table 1. Activity distribution in concentrate and filtrate after ultrafiltration of the two thioltransferase components. The most acidic (peak I) and the most basic (peak II) components were separated by CM-cellulose chromatography and were subjected to ultrafiltration as described in Materials and methods.

Filter	Activity ($10^3 \times$ units/ml) Peak I Concentrate (C)	Peak I Filtrate (F)	$\frac{F}{C}$	Peak II Concentrate (C)	Peak II Filtrate (F)	$\frac{F}{C}$
PM 30	99	0	0	1232	0	0
XM 50	332	7.5	0.02	2304	30	0.01
XM 100	504	116	0.23	4192	896	0.21

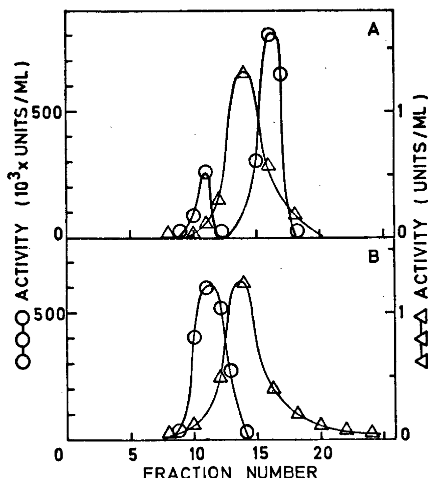

Fig. 2. CM-cellulose chromatography of (A) an untreated control and (B) a GSH-treated rat liver supernatant. The supernatant fraction from 30 g of rat liver was adjusted to pH 8.0 and divided into two parts. One of these was used as a control in the subsequent CM-cellulose chromatography. The second part was treated with 2 mM GSH for 5 min. Both parts were chromatographed simultaneously on separate Sephadex G-25 columns to change the solvent to that used in the subsequent CM-cellulose chromatography. The symbols for enzymatic activities are given in the legend to Fig. 1.

exclude an oligomer-monomer transition as the explanation for the two peaks of thioltransferase activity.

Glutathione, which is present in high concentration in rat liver,[5] is a substrate of the thioltransferase and has been found to activate aged preparations of the enzyme (Ref. 6 and unpublished results). Therefore, another explanation of the appearance of two thioltransferase components could be that the enzyme occurs in both a GSH-containing and a GSH-deficient form. Fig. 2 illustrates an attempt to explore this possibility. It is shown that after incubation with 2 mM GSH (see MATERIALS AND METHODS), the second component, after chromatographing on CM-cellulose had disappeared and the first component had increased. The first component was identified from its position in relation to that of glutathione reductase in the elution profile. The position of glutathione reductase in the elution profile was not affected by pretreatment with GSH, nor could any effect on the positions of the two peaks of aryltransferase be demonstrated (*cf.* Ref. 1). This conclusion was based on measurements of the pH, volume, and conductance of the effluent. Furthermore, the GSH-treated thioltransferase had a total activity corresponding to the sum of the activities of the two peaks obtained from the untreated control sample, which supported the interpretation that the second component was converted into the first one.

To obtain further evidence for this interpretation, the following experiment was carried out. A sample was divided into three equal parts, one of which served as an untreated control for CM-cellulose chromatography. The remaining two parts were treated with 2 mM GSH as in the experiment described above, and one of these was afterwards incubated with 1 mM DTE before the ion-exchange chromatography. The purpose of the experiment was to confirm the previous observation and to find out whether the effect of GSH could be reversed by DTE, which is a strong disulfide-reducing agent. The amount of DTE used was sufficiently small to prevent complete conversion of the first component into the second one. This precaution was taken to eliminate the possibil-

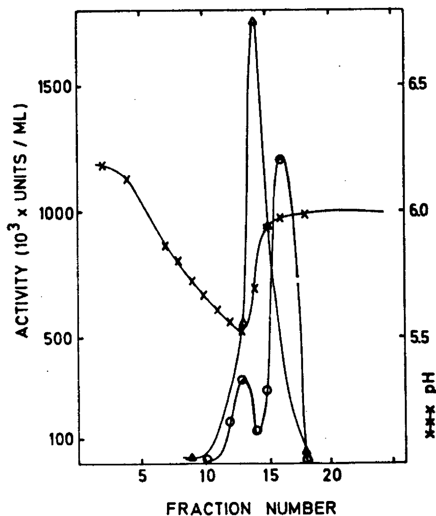

Fig. 3. The reversal by dithioerythritol (DTE) of the effect of GSH-treatment seen in Fig. 2. GSH-treated sample (see Materials and methods and Fig. 2) was incubated with 1 mM DTE for 5 min at pH 8.0 before the subsequent Sephadex G-25 and CM-cellulose chromatography. Thioltransferase (activity × 2). The symbols for enzymatic activities are given in the legend to Fig. 1.

ity that the effect of the DTE-treatment was a change in the relative positions of glutathione reductase and the first thioltransferase peak. The three different preparations were freed by gel filtration from the reagents used in the pretreatment and were chromatographed under identical conditions on CM-cellulose columns of equal sizes. The results of the chromatography of the untreated and GSH-treated samples were similar to those described in Fig. 2; and the result obtained with the DTE-treated preparation demonstrated a reappearance of the second thioltransferase peak (Fig. 3) such that this peak contained an even greater amount of the total thioltransferase activity than the second peak from the untreated control. The chromatographic properties of glutathione reductase were not affected by the DTE-treatment. Thus, this experiment demonstrates that the second component can be converted into the more acidic one by treatment with GSH and that the more acidic component can then be reconverted into the second one by incubation with DTE.

To obtain direct evidence that GSH was bound to the thioltransferase, [^{35}S]-GSH was incubated with different enzyme preparations Treatment of the supernatant fraction of rat liver with [^{35}S]-GSH resulted in only a small incorporation of radioactivity, because the concentration of endogenous GSH in the supernatant was approximately 1 mM, which was in large excess of the [^{35}S]-GSH added. Furthermore, extensive washing of the material adsorbed onto CM-cellulose released [^{35}S]-GSH in a large volume after the effluent containing the unadsorbed protein. This leakage may arise from dissociation of GSH from the enzyme-GSH complex. Consequently, no distinct peak of radioactivity was obtained on subsequent elution with a linear salt gradient.

However, the results were more definitive when the basic form of thioltransferase obtained by CM-cellulose chromatography was treated with radioactive GSH and rechromatographed on CM-cellulose in order to visualize [^{35}S]-GSH binding to thioltransferase. This treatment demonstrated the formation of an acidic thioltransferase which coincided with the peak of radioactivity in the elution profile (Fig. 4). Coincidence between enzyme activity and radioactivity is in itself not conclusive evidence for a

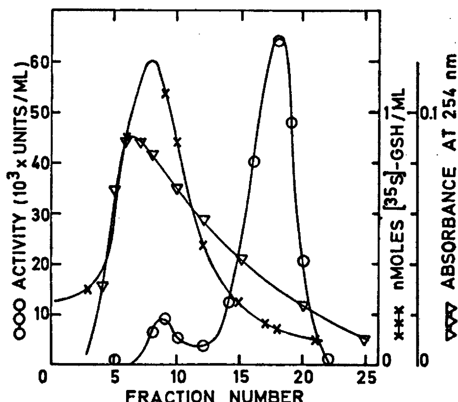

Fig. 4. Incorporation of [^{35}S]-GSH into the basic thioltransferase component. The basic component (22 ml, 1.64 mg protein per ml) obtained after CM-cellulose chromatography was adjusted to pH 8 and incubated with 120 μM [^{35}S]-GSH (spec. act. 5 Ci/mol). The incubation mixture was chromatographed on Sephadex G-25 and then chromatographed on a CM-cellulose column (2 x 4 cm). Unadsorbed proteins and free GSH were washed out before elution of the thioltransferase with a linear salt gradient. The gelfiltration removed 60 % of the original radioactivity, 7 % was recovered in the effluent (50 ml) preceding the gradient, and 0.5 % appeared in the fractions containing the acidic form of the thioltransferase. The symbols are: ▽, absorption at 254 nm; ×, [^{35}S]-GSH; O, CySSG-thioltransferase.

GSH-enzyme complex, because the enzyme species in question is not pure enough. However, this evidence for the binding of [^{35}S]-GSH to thioltransferase is strengthened by the fact that incubation of the basic form of thioltransferase with radioactive GSH gives rise to an acidic enzyme form and a coincident peak of radioactivity.

These results support the hypothesis that the enzyme can exist in both a GSH-containing and a GSH-deficient form, as does the finding that the GSH-treatment gives rise to the most acidic thioltransferase component, which is expected due to the acidic properties of GSH. This explanation is also in accord with the results of the ultrafiltration experiments in which no significant difference in the molecular weights of the two components could be demonstrated.

It was also shown by equilibrium dialysis that GSH was bound to a partially purified thioltransferase preparation, but the possibility that the

binding measured was caused by components other than the thioltransferase could not be excluded.

It is assumed that GSH is covalently linked to the enzyme *via* a disulfide bond. If so, DTE-treatment of the native enzyme would be expected to increase the second component at the expense of the first one, a result which was in fact obtained experimentally. The binding of GSH to the enzyme could take place by thiol-disulfide interchange either between GSH and a disulfide group of the enzyme or between GSSG (formed by oxidation of GSH) and a sulfhydryl group of the protein. To test the latter possibility, the enzyme was treated with 1 mM GSSG before chromatography on CM-cellulose. This treatment gave no significant increase of the more acidic component, which lends support to the former mechanism of binding. According to this interpretation, GSH and a disulfide-containing form of the thioltransferase are in equilibrium with a mixed disulfide of GSH and the enzyme (Fig. 5). This scheme explains why the amount of the GSH-containing component decreases with the size of the chromatographic bed in the gel filtration experiments; removal of GSH shifts the equilibrium in favor of the GSH-deficient enzyme form. It should also be pointed out that the binding of GSH according to Fig. 5 may be a partial reaction of the catalytic mechanism of the enzyme.

It is noteworthy that glutathione *S*-epoxide transferase can also occur in multiple forms and that these forms are also interconvertible under the influence of GSH.[7] Furthermore, the GSH concentration influences the relative proportions of the three major components of glucose-6-phosphate dehydrogenase in rat liver.[8] Highly purified enzymes, such as glyceraldehyde-3-phosphate dehydrogenase[9] and acylphosphatase,[10] have been found to contain bound GSH, but in these cases the possibility exists that these enzyme forms are artifacts formed during purification. Data in the literature also suggest that enzymatic activities can be modulated by formation of mixed disulfides with low-molecular-weight compounds (*cf.* Refs. 11 – 13) and that GSH can be involved in such thiol-disulfide interchange reactions. This postulated physiological function receives support from the finding that a substantial proportion of the cellular GSH is bound to proteins.[14–16]

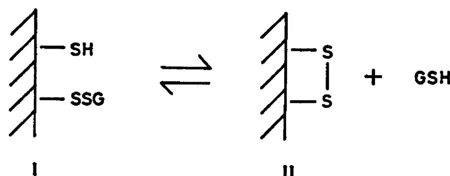

Fig. 5. The proposed equilibrium between GSH and CySSG-thioltransferase. The enzyme forms I and II correspond to the first (acidic) and second (basic) component, respectively, obtained after chromatography on CM-cellulose.

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