The purification of the hepatic glutathione *S*-transferases of rainbow trout by glutathione affinity chromatography alters their isoelectric behaviour

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1. The basic glutathione *S*-transferases from rainbow-trout liver were more stable than the acidic ones. 2. The apparent pI values of these enzymes were lowered when they were eluted from a glutathione affinity column by reduced glutathione at pH 8.85. 3. The pI effect was not a function of the high pH alone, was diminished under conditions less favourable to glutathione oxidation, and did not occur when *S*-hexylglutathione affinity chromatography was used instead.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of multifunctional detoxification enzymes with a wide distribution in the animal kingdom (Jakoby, 1978; Baars & Breimer, 1980). The enzymes in rat liver cytosol have been studied in much greater detail than those from other sources, and considerable structural information is available about them. They are dimeric proteins, each comprising two of at least four subunits, termed Ya (M_r 22000), Yb (M_r 23500), Yb' (M_r 23500) and Yc (M_r 25000) (Bass *et al.*, 1977; Mannervik & Jonsson, 1982). These dimers can be resolved from one another by ion-exchange chromatography (Hayes *et al.*, 1979) and chromatofocusing (Jonsson *et al.*, 1982); at least seven separate enzymes have been identified by the use of these techniques.

A powerful and popular method for the partial purification of the glutathione *S*-transferases is affinity chromatography, with the use of columns developed with either reduced glutathione (GSH) (Simons & Vander Jagt, 1977) or *S*-hexylglutathione (Mannervik & Guthenberg, 1981). Transferases from guinea-pig liver (Irwin *et al.*, 1980), human liver (Awasthi *et al.*, 1980) and bovine ocular lens (Saneto *et al.*, 1980) have been purified with the former, and from rat liver with the latter.

We have used both columns in a study of the hepatic glutathione *S*-transferases from rainbow trout (*Salmo gairdnerii*), and now demonstrate that the elution of the enzymes from the glutathione column alters their behaviour on subsequent cation-exchange chromatography. By contrast, the *S*-hexylglutathione column does not change the properties of the enzymes so far as cation-exchange chromatography is concerned.

Abbreviation used: GSH, reduced glutathione.

Experimental

Materials

1-Chloro-2,4-dinitrobenzene and 2-mercaptoethanol were purchased from BDH Chemicals (Poole, Dorset, U.K.), and *N*-acetyl-L-cysteine was from Boehringer Corp. (Lewes, East Sussex, U.K.). CM-Sephadex C-50 was from Pharmacia (Hounslow, Middx., U.K.). DL-Dithiothreitol, GSH and epoxy-activated Sepharose 6B were all supplied by Sigma Chemical Co. (Poole, Dorset, U.K.).

Fish

Rainbow trout (100–300 g) were collected from a local trout farm (Penicuik Trout Farm, Penicuik, Midlothian, Scotland, U.K.).

Preparation of cytosol

Fish were killed by cervical dislocation, and their livers were perfused *in situ* with ice-cold 154 mM-NaCl. These were then removed, chopped and homogenized in 4 vol. of ice-cold 22 mM-potassium phosphate buffer, pH 7.4, in a motor-driven glass/Teflon homogenizer. Cytosol was prepared by centrifuging the homogenate [9000 g (r_{av} 7.0 cm) for 25 min at 4°C followed by 100 000 g (r_{av} 5.95 cm) for 80 min at 4°C].

Analytical methods

Glutathione *S*-transferases activity was assayed spectrophotometrically at 25°C at 340 nm, with 1 mM-GSH and 1 mM-1-chloro-2,4-dinitrobenzene as substrates (Habig *et al.*, 1974; Nimmo *et al.*, 1979). The buffer was 100 mM-potassium phosphate, pH 6.5, and the reaction volume was 3 ml.

CM-Sephadex chromatography

Cytosol or partially purified enzyme preparations were dialysed (for 20 h at 4°C) against two changes of 20 vol. of CM buffer (10 mM-sodium phosphate buffer, pH 7.5, containing 1 mM-*N*-acetylcysteine). When partially purified enzyme was being applied after affinity chromatography, the CM buffer also contained 10 mM-2-mercaptoethanol. The non-diffusible material (15 ml) was applied to a column (2.2 cm × 15 cm) of CM-Sephadex C-50 equilibrated and eluted with CM buffer (38.4 ml/h). After 35 fractions (2.5 ml) had been collected, a linear gradient of NaCl was applied as previously described (Strange *et al.*, 1977).

Glutathione affinity chromatography

Cytosol (about 70 ml) was applied to a glutathione-Sepharose 6B affinity column (1 cm × 10 cm) prepared by the method of Simons & Vander Jagt (1977), equilibrated with 22 mM-phosphate (16 mM-KH₂PO₄ + 28 mM-Na₂HPO₄) buffer, pH 7.0. The column was developed with 50 ml of 100 mM-Tris, pH 9.6 (24 ml/h), followed by 200 ml of Tris/GSH (100 mM-Tris containing 10 mM-GSH, final pH 8.85). Fractions (5 ml) were collected and pooled. The pH values of all solutions were measured at 25°C.

Glutathione affinity chromatography with the use of deoxygenated buffers

The procedure differed from the above as follows. The phosphate buffer and the water used to prepare the Tris solutions were boiled, and then DL-dithiothreitol was added to 10 mM. The Tris solution was titrated with HCl to pH 8.6, and had GSH added to 10 mM immediately before use, lowering the pH to 8.4. All the solutions were gassed with N₂ and stored in air-tight vessels.

S-Hexylglutathione affinity chromatography

Cytosol (about 70 ml) was applied to an S-hexylglutathione-Sepharose 6B affinity column (1 cm × 30 cm) and eluted (38.4 ml/h) with S-hexylglutathione at pH 7.8 as described by Mannervik & Guthenberg (1981). Fractions (5 ml) were collected, pooled and treated in the same way as those from the glutathione affinity column. No special precautions were taken to deoxygenate the buffers used.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed as described previously (Laemmli, 1970; Nimmo *et al.*, 1981), with minor modifications.

Results

CM-Sephadex ion-exchange chromatography

The elution profile for cytosol that had been dialysed overnight is shown in Fig. 1(a). It demonstrates that the activity of the acidic transferases in fresh trout liver cytosol is greater than that of the basic transferases. The broad peak of acidic transferases (fractions 7–45) amounted to 77% of the total activity, the basic transferases being eluted in fractions 64–80. When identical cytosol was dialysed for 80 h and then applied to the column

Fig. 1. Elution profile from CM-Sephadex C-50 of (a) trout liver cytosol, (b) trout liver cytosol after extended dialysis against CM buffer, and (c) material partially purified by glutathione affinity chromatography and dialysed overnight against CM buffer

Cytosol or preparation derived therefrom was dialysed against CM buffer (see the text). Non-diffusible material (15 ml) was then applied and eluted with CM buffer (38.4 ml/h). After 35 fractions (2.5 ml) had been collected, a linear salt gradient (0–500 mM-NaCl) was applied (arrow). Glutathione S-transferase activity (μmol of substrate conjugated/min per ml of fraction) was assayed with 1-chloro-2,4-dinitrobenzene as substrate.

(Fig. 1*b*), the acidic transferases accounted for 53% of the total activity. The change in the relative proportions of acidic and basic transferases is due mainly to a decrease in the activity of the acidic transferases and partly to a slight increase in that of the basic transferases. Although the reason for this increase has not been determined, there was no such increase in A_{280} for the basic transferase peak (not shown), implying that the change was not due to acidic transferases being eluted as basic ones. In contrast, when cytosol that had first been partially purified by glutathione affinity chromatography was eluted from the CM-Sephadex column (Fig. 1*c*), all the activity appeared in the acidic transferase fractions (6–21). Extended dialysis (48 h) of this material produced a similar profile (not shown), but with a slight broadening of the acidic transferase peak and a very small basic transferase peak.

To determine whether the pattern of Fig. 1*c* was linked to the pH of elution (8.85) or to the presence of GSH at that pH, conditions were created to simulate elution. When cytosol (50 ml; dialysed to remove endogenous GSH) was sequentially re-dialysed against Tris/GSH (100 mM-Tris containing 10 mM-GSH; 25 vol.; 18 h) followed by CM buffer (25 vol.; two changes; 24 h) and then eluted from the ion-exchange column, the profile (Fig. 2*a*) had both acidic and basic transferases, the latter comprising only 16% of the total activity. The profile is intermediate between those of Figs. 1*b* and 1*c*, with the acidic transferases showing less retardation than those in Fig. 1*b*. However, when the Tris/

GSH solution was replaced with Tris (100 mM; pH 9.6), the basic transferases comprised 54% of the total activity (Fig. 2*b*). This profile closely resembles that for stored cytosol (Fig. 1*b*) and suggests that the pattern of Fig. 1*c* is due to the presence of GSH at a pH of 8.85 and not to pH alone.

To find out if the effect of GSH at an alkaline pH is connected with the oxidation of GSH to its disulphide under the conditions employed with the elution from the glutathione affinity column, the elution was repeated with deoxygenated buffers at a slightly lower pH and with the presence of a thiol reducing agent (DL-dithiothreitol). The profile (not shown) was very similar to that in Fig. 1*a*. The acidic transferases appeared as a broad peak (fractions 7–50), and not as a sharp one as occurred when untreated buffers were used (Fig. 1*c*). The basic transferases were eluted as a sharp peak (fractions 58–75) similar in magnitude to that in Fig. 1*b*. The acidic transferase peak constituted over 80% of the total activity. When cytosol partially purified by using *S*-hexylglutathione affinity chromatography was applied to the ion-exchange column, a sharp peak of basic transferase activity (accounting for 72% of the total activity) and a smaller peak of acidic transferase activity (showing some degree of retardation) were eluted (not shown). The profile showed a strong similarity to that of Fig. 1*b*.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels of samples before and after glutathione affinity chromatography showed that the most acidic transferases had components that migrated at essentially the same rates as the Ya and Yb subunits of rat liver glutathione *S*-transferases, whereas the remaining transferases had only the component migrating at the same rate as the Ya subunit. The subunit composition of the transferases was not changed by glutathione affinity chromatography or *S*-hexylglutathione affinity chromatography.

Discussion

When the glutathione *S*-transferases from trout liver are stored before ion-exchange chromatography, the relative proportion of the acidic transferases decreases. If this is true for other species as well, it implies that the relative activities of their transferases will depend on the time taken to separate them. In contrast, when glutathione *S*-transferases are purified by glutathione affinity chromatography under standard conditions, their isoelectric points apparently become more acidic (see Fig. 1*c*). A comparison of Figs. 1*b* and 1*c*

Fig. 2. Elution profile from CM-Sephadex of trout liver cytosol after dialysis against (a) Tris/GSH or (b) Tris. Cytosol (after initial dialysis against CM buffer) was dialysed first against Tris/GSH or Tris and then overnight against CM buffer before application. Otherwise treatment was identical with that indicated in Fig. 1.

reveals that the activity eluted entirely as acidic transferases (Fig. 1c) is very close to the total acidic and basic transferase activity eluted after dialysis (Fig. 1b). In contrast, transferases partially purified by *S*-hexylglutathione affinity chromatography did not behave anomalously on subsequent ion-exchange chromatography.

A possible explanation for the apparent decrease in isoelectric points of the transferases purified by glutathione affinity chromatography is that the enzymes were deamidated under the alkaline conditions. This was discounted because the fall in pI values was observed in proteins dialysed against Tris/GSH (pH 8.85), but not in proteins dialysed against Tris alone (pH 9.6). A second possible explanation is that the glutathione anion forms a mixed disulphide with the transferases. Either GSH could be oxidized to its disulphide at the alkaline pH employed and then react with thiol groups in the proteins, or GSH could give the mixed disulphides directly by reacting with cystine residues in the proteins. In either event the protein would gain one extra negative charge with each molecule of GSH incorporated. The experiment with deoxygenated buffers suggested that oxygen is a factor in the reaction, and that oxidized glutathione is the reactive intermediate. Co-purification of the enzyme glutathione thioltransferase (Mannervik & Eriksson, 1973) by glutathione affinity chromatography could further promote the reaction. On the other hand *S*-hexylglutathione would not react in this way, as its thiol group is blocked.

Although we have studied only the trout glutathione *S*-transferases, the observed change in the chromatographic properties of these enzymes may have a much wider significance. In a number of studies of the glutathione *S*-transferases, glutathione affinity chromatography has been used before pI determinations (Awasthi *et al.*, 1980; Irwin *et al.*, 1980; Nishiya *et al.*, 1981) and ion-exchange chromatography (Saneto *et al.*, 1980; Simons & Vander Jagt, 1980). If this effect occurs to the same extent with the glutathione *S*-transferases from other species, then a number of pI values and ion-exchange profiles may have to be re-evaluated. Likewise, the properties of commercially prepared glutathione *S*-transferases (from bovine, horse, pig, rabbit and rat livers; Sigma Chemical Co., 1983) may differ from those of the enzymes *in vivo*.

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