PURIFICATION AND BILIRUBIN BINDING PROPERTIES OF GLUTATHIONE *S*-TRANSFERASE FROM HUMAN PLACENTA

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1. Introduction

The liver glutathione *S*-transferases (EC 2.5.1.18) are a family of closely related enzymes which have an important role in the detoxification of xenobiotics [1–3]. They also function as intracellular transport proteins for the removal of exogenous and endogenous compounds including bilirubin. In [4,5] glutathione *S*-transferase was isolated from human placenta. Placenta glutathione *S*-transferase is an acidic protein (pI 4.6–4.8) whereas the multiple transferases found in liver are basic proteins [6,7]. The transport role of placenta glutathione *S*-transferase, especially its role in bilirubin transport, has not been evaluated. We report here on a convenient one-step purification of glutathione *S*-transferase from human placenta using glutathione–affinity chromatography. Data are also presented which indicate that placenta glutathione *S*-transferase binds bilirubin at a site which is distinct from the catalytic site.

2. Materials and methods

1-Chloro-2,4-dinitrobenzene was purchased from Aldrich. GSH and bilirubin were obtained from Sigma. Solutions of bilirubin were prepared daily in 0.1 M NaOH. The GSH–affinity matrix was prepared by coupling GSH to epoxy-activated Sepharose as in [8,9]. *S*-Methylglutathione was prepared from GSH and methyliodide [10].

Measurements of glutathione *S*-transferase activity were carried out at 25°C in 0.02 M potassium phosphate buffer (pH 6.5) containing 0.1 M NaCl, 2.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene and 4% ethanol to solubilize this substrate. The reaction was followed at 340 nm, corrected for the background reaction. Activity is in units μmol/min. One unit of activity corresponds to an absorbance change of 3.2 *A*/min at 340 nm in a 3 ml reaction volume, 1 cm pathlength, assuming $\Delta\epsilon = 9.6\ mM^{-1} \cdot cm^{-1}$ [11].

The binding of bilirubin to placenta glutathione *S*-transferase was determined from analysis of the quenching of the intrinsic protein fluorescence upon binding bilirubin. K_d was obtained from plots of:

$$\log \frac{\Delta F_c}{F_o - \Delta F_c} \text{ vs } \log [br]$$

where F_o is the maximum quenching and ΔF_c are the quenching values corrected for inner filter effects. Measurements were carried out in 0.02 M potassium phosphate buffer (pH 6.5) containing 0.1 M NaCl; $\lambda_{ex} = 280$ nm, $\lambda_{em} = 320$ nm, slits = 6 nm.

Protein was determined by the method in [12].

3. Results

3.1. *Purification of glutathione* S-*transferase from human placenta*

We had utilized GSH–affinity chromatography to purify glutathione *S*-transferase from liver [8,9]. The same procedure was applied to glutathione *S*-transferase from human placenta. Two fresh placenta (800 g) were homogenized in 0.02 M Tris/Tris–HCl (pH 7.2) containing 0.25 M sucrose and 0.1 mM EDTA. The homogenate was centrifuged at 24 000 × *g* for 30 min, and then the supernatant was centrifuged at 100 000 × *g* for 1 h giving 1050 ml starting material for protein purification. The 100 000 × *g* supernatant contained 38 mg protein/ml and showed glutathione *S*-transferase spec. act. 0.2 units/mg. In a typical purification, ~400 units of activity was added to a GSH-affinity column (1 × 15 cm) which was equilibrated with 0.01 M potassium phosphate buffer.

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The column was washed with this buffer until no protein appeared in the effluent. The column then was washed with 0.05 M Tris, pH 9.6 (4°C), containing 5 mM GSH. Glutathione *S*-transferase was removed from the column with this buffer. The recovered enzyme showed spec. act. 136 units/mg, representing a 680-fold purification. Recoveries of activity were 85–95%.

A sample of glutathione *S*-transferase purified as above was purified further by hydroxylapatite chromatography. Glutathione *S*-transferase, 160 units, was added to a hydroxylapatite column (1 × 5 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0) containing 5 mM GSH. The column was developed with a phosphate gradient from 0.01–0.3 M in 100 ml total volume with 5 mM GSH present. Glutathione *S*-transferase was removed as a single peak of activity (97% recovery). The specific activity of the recovered transferase was the same as that of the enzyme recovered from the affinity column.

Glutathione *S*-transferase purified by these two procedures gave single bands on SDS–PAGE with subunit M_r 22 000. Thus, the transferase from human placenta appears to be homogeneous after the one-step purification by GSH–affinity chromatography. Rabbit antiserum was raised against purified placenta glutathione *S*-transferase. There was no cross reactivity between human liver and placenta transferase on crossed immunoelectrophoresis.

3.2. *Bilirubin binding to placenta glutathione S-transferase*

The quenching of the intrinsic protein fluorescence was followed upon addition of bilirubin to 1 μM solutions of placenta glutathione *S*-transferase (pH 6.5). The experimental points were corrected for inner filter effects. The corrected fluorescence values were plotted (fig.1, K_d = 14 μM). The experiment was repeated in the presence of 2.5 mM GSH. In the presence of GSH, the dissociation constant for the binding of bilirubin to placenta glutathione *S*-transferase is 23 μM. These values are somewhat higher than the dissociation constants determined for the binding of bilirubin to glutathione *S*-transferase from human liver [13]. The main difference, however, is the lack of cooperativity observed with placenta glutathione *S*-transferase. Liver transferase, by comparison, shows positive cooperativity for the binding of bilirubin with a Hill coefficient of 1.5 and a composite $K' \sim 5$ μM.

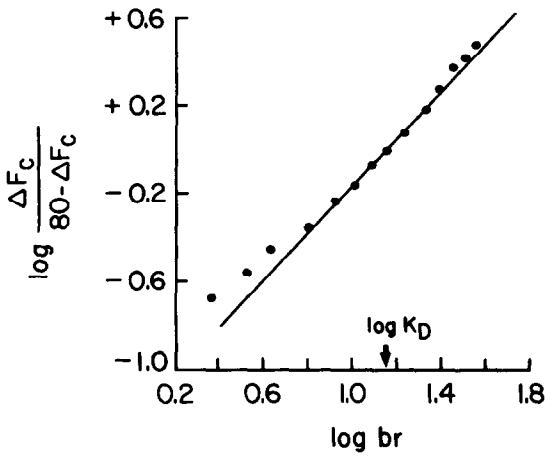

Fig.1. Plot of the fluorescence quenching data for the addition of bilirubin to glutathione *S*-transferase from human placenta. Bilirubin was added to transferase (1 μM) at pH 6.5, 25°C, in the absence of GSH K_d = 14 μM. In the presence of GSH (2.5 mM) K_d = 23 μM.

The effects of bilirubin on the catalytic activity of placenta glutathione *S*-transferase were evaluated. Transferase was added to assay mixtures of 2.5 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene containing up to 43 μM bilirubin. There was no inhibition of the transferase activity by the bilirubin. Owing to solubility limits, higher concentrations of bilirubin were not used. It is clear, however, that the transferase activity of placenta glutathione *S*-transferase is not affected by bilirubin at concentrations sufficient to quench most of the protein fluorescence. We conclude that placenta glutathione *S*-transferase binds bilirubin at a site which is distinct from the catalytic site.

3.3. *Multiple activity states of placenta glutathione S-transferase*

The kinetic properties of placenta glutathione *S*-transferase depend upon the presence of GSH and are time dependent, suggesting that slow conformational changes are involved (fig.2). In the presence of 2.5 mM GSH, glutathione *S*-transferase is stable in its high activity state. In the absence of GSH, glutathione *S*-transferase adopts a state of intermediate activity. Substitution of *S*-methylglutathione (2.5 mM) for GSH kept glutathione *S*-transferase in its high activity state. *S*-Methylglutathione is a competitive inhibitor of GSH in the transferase reaction (K_i = 0.1 mM). Cysteine or *N*-acetylcysteine, however, did not

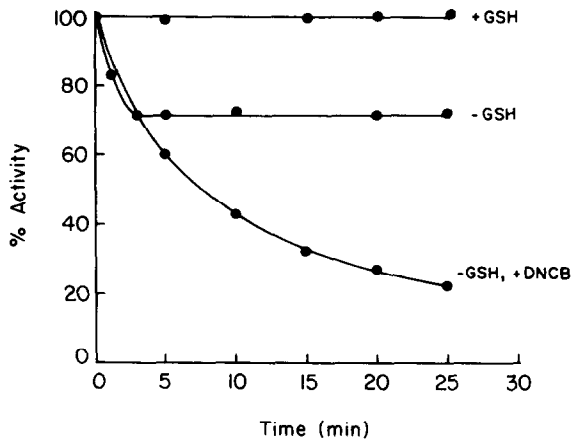

Fig.2. Multiple activity states of glutathione *S*-transferase from human placenta. GSH (or a GSH analog) is required to keep the enzyme in a high activity state. In the absence of GSH, glutathione *S*-transferase adopts a state which has intermediate activity. In the presence of the substrate 1-chloro-2,4-dinitrobenzene (DNCB), transferase adopts a state which shows low activity. Analogs of GSH protect against this effect of the substrate. All activities were determined at pH 6.5, 25°C.

prevent glutathione *S*-transferase from adopting the state with intermediate activity.

In the presence of the substrate 1-chloro-2,4-dinitrobenzene, glutathione *S*-transferase adopts a state with low activity (fig.2). Again, *S*-methylglutathione protected against this loss of activity. Thus, it appears that GSH or a GSH analog is required to keep placenta glutathione *S*-transferase in a conformation which exhibits high catalytic activity.

4. Discussion

This study was undertaken for 2 reasons:

(1) We wished to evaluate more fully the glutathione-affinity procedure which we had used in our earlier studies of liver glutathione *S*-transferase. Liver transferase is a family of proteins. In our earlier work, these multiple forms were first separated, and the affinity procedure was used for the final step of the purification. Consequently, the usefulness of the affinity procedure for a one-step purification of glutathione *S*-transferase was not apparent. Here, we have demonstrated the usefulness of this affinity procedure for the one-step purification of placenta glutathione *S*-transferase to apparent homogeneity.

(2) We wished to evaluate the bilirubin binding properties of placenta glutathione *S*-transferase. There have been no reports of specific bilirubin binding proteins in placenta. This study suggests that placenta glutathione *S*-transferase not only binds bilirubin but does so at a site distinct from the transferase catalytic site. Given that a typical placenta contains 20–30 mg glutathione *S*-transferase, one could conclude that glutathione *S*-transferase is a major bilirubin transport protein in the placenta.

The multiple glutathione *S*-transferases from human liver also bind bilirubin [7,13]. This binding is complex and appears to involve multiple sites [13]. The transferase activity of the liver glutathione *S*-transferases is sensitive to the binding of bilirubin, unlike the situation with the placenta enzyme. The liver and placenta enzymes exhibit fundamentally different properties. Thus, it is not surprising that they are immunologically distinct.

Acknowledgement

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