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Tissue distribution and subunit structures of the multiple forms of glutathione *S*-transferase in the rat

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A study of the subunit structures of the multiple forms of glutathione *S*-transferase in rat kidney, testis, lung and spleen is shown to be consistent with a proteolytic model for the generation of the multiple forms.

Rat liver glutathione *S*-transferase (EC 2.5.1.18) is a dimeric protein of mol.wt. 45000 that exists in several chromatographically separable forms. The most studied forms C, B, A and AA bind to CM-cellulose at pH 6.7 and are eluted in that order with a 0–75 mM-KCl gradient (Habig *et al.*, 1974). Early work suggested that these forms were made up of similar subunits of mol.wt. 25000 (Habig *et al.*, 1974). However, only forms A and C show similar immunological determinants (Habig *et al.*, 1974; Guthenberg & Mannervik, 1979), so that the relationship between these and the other rat forms remains obscure.

The glutathione *S*-transferases play an important role in the detoxification of xenobiotics containing an electrophilic centre (see Jakoby, 1978) and, in addition, are quantitatively important intracellular binding proteins that have been postulated to regulate the flux of bilirubin, cholic acid and other organic anions from plasma into the liver (Levi *et al.*, 1969*a*,*b*; Reyes *et al.*, 1971; Fleischner *et al.*, 1972).

A suggestion (Bass *et al.*, 1977) that the glutathione *S*-transferases are dimers of three possible subunits c (mol.wt. 25000), b (mol.wt. 23500) and a (mol.wt. 22000) has been confirmed for the rat liver forms C, B, A and AA, which have been identified as bb, ac, bb and cc respectively (Hayes *et al.*, 1980; Scully & Mantle, 1980). The high-affinity binding site for lithocholate (Hayes *et al.*, 1979) and sulphobromopthalein (Bhargava *et al.*, 1980*a*) is associated with the a-subunit, whereas high-affinity sites for cholic acid are associated with a- and c-subunits (Hayes *et al.*, 1980). The question of the origin of the multiple forms of the glutathione *S*-transferases therefore relates to a tissue's specificity and binding capacity for organic anions. A previous suggestion that deamidation is responsible for generating the multiple forms of human liver glutathione *S*-transferase (Kamisaka *et al.*, 1975) is clearly not applicable to the situation in rat liver, with the possible exception of forms C and A (Scully & Mantle, 1980). The identification of glutathione *S*-transferase AA as a dimer of c-subunits (Hayes *et al.*, 1980; Scully & Mantle, 1980) is consistent with the hypothesis that the multiple forms arise as a result of proteolytic action on a cc-homodimer (Hayes *et al.*, 1979). We present further evidence for a proteolytic mechanism based on an analysis of the subunit structures of the major forms from several extrahepatic tissues of the rat.

Abbreviation used: SDS, sodium dodecyl sulphate.

Materials and methods

Purification of the various forms of glutathione S-transferase

Male Wistar rats were killed by a blow on the head, exsanguinated and the tissues removed and homogenized in 4 vol. of 250 mM-sucrose/10 mM-sodium phosphate/2 mM-EDTA, pH 7.2. The homogenate was centrifuged at 48000 ***g*** for 30 min, the supernatant was immediately gel-filtered into 10 mM-sodium phosphate, pH 7.2, and applied to a DEAE-cellulose column (20 cm × 2.5 cm) pre-equilibrated in the same buffer. Protein-containing fractions eluting with the loading buffer were titrated to pH 6.7 with 1 M-acetic acid and loaded on to a CM-cellulose column (20 cm × 2.5 cm) pre-equilibrated with 10 mM-sodium phosphate, pH 6.7. Forms D and E were eluted with the running buffer and a 0–75 mM-KCl gradient (2 × 500 ml) was then applied to fractionate forms C, B, A, and AA. The various forms were then further purified by gel filtration using Sephadex G-100 equilibrated with 10 mM-sodium phosphate/100 mM-KCl, pH 7.2. The fractions from CM-cellulose chromatography of liver cytosol containing transferases D and E were adjusted to pH 6, loaded on to CM-cellulose pre-

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equilibrated with 10mM-sodium phosphate, pH6, and resolved with a linear KCl gradient (0–150mM; 2 × 400ml).

SDS/polyacrylamide-gel electrophoresis

Fractions from CM-cellulose chromatography or gel filtration on Sephadex G-100 were precipitated with 10% (w/v) trichloroacetic acid, washed twice with acetone and taken up in running buffer as described by Maizel (1971). SDS/polyacrylamide-gel electrophoresis was carried out by using the system of Maizel (1971) as modified by Thomas (1978).

Glutathione S-transferase activity

Enzyme activity was assayed by using 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene (a substrate that is particularly active with forms A and C) as substrates (Habig *et al.*, 1974).

Results and discussion

Every tissue examined (liver, kidney, spleen, testis and lung) exhibited glutathione *S*-transferase activity that did not bind to CM-cellulose at pH6.7. This fraction, which contains both forms D and E in liver, was not further characterized for the extrahepatic tissues in the present study.

Results of CM-cellulose chromatography of kidney cytosol at pH6.7 are shown in Fig. 1(*a*). In addition to forms B and AA (Scully & Mantle, 1980), there is an additional form, X, that we have now isolated in three out of six preparations (see also Hales *et al.*, 1978). The assignment of the peaks is based on a comparison of the conductivity of the eluant with the liver chromatogram as a reference, substrate specificity (i.e. no activity of any kidney form with 1,2-dichloro-4-nitrobenzene) and also that the kidney forms B and AA co-elute with the liver forms B and AA on rechromatography (results not shown). The 'variable' form, X, is not form C, since it shows no activity with 1,2-dichloro-4-nitrobenzene. Fig. 1(*b*) shows the results of a similar experiment using testis cytosol. The identity of forms A and C is confirmed by their ability to utilize 1,2-dichloro-4-nitrobenzene as a substrate. The slight shoulder on form C seen when 1-chloro-2,4-dinitrobenzene was used as a substrate is probably due to a trace of form B (see below).

The results of SDS/polyacrylamide-gel electrophoresis over the peak fractions of kidney forms B and AA and testis forms C, A and AA are shown in Fig. 2. The subunit compositions are entirely consistent with those previously reported for liver (Scully & Mantle, 1980). In addition, fraction 17 from the testis chromatogram (see Fig. 1*b*) reveals traces of form B (the ac-heterodimer) in the shoulder of peak C. Examination of Fig. 2(*a*) reveals that

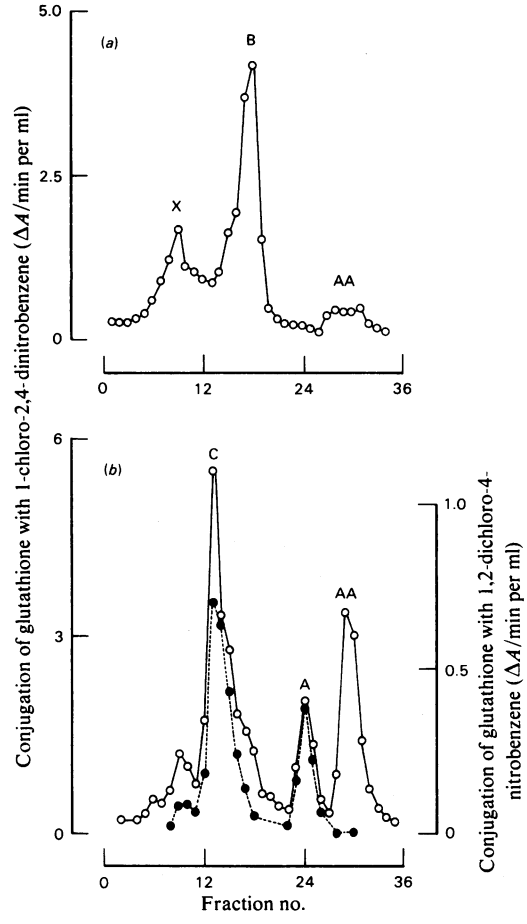


Fig. 1. *CM-cellulose chromatography of kidney (a) and testis (b) cytosol at pH6.7*

Enzyme activity was assayed with 1-chloro-2,4-dinitrobenzene (○) and 1,2-dichloro-4-nitrobenzene (●) as substrates. The salt gradient starts at fraction 1; other conditions are described in the text.

kidney form X is a homodimer of a-subunits [i.e. equivalent to the definition of ligandin as described by Hayes *et al.* (1979)]. Since this form is only seen occasionally, we have examined the possibility that ligandin (i.e. the aa-homodimer) is not a major form *in vivo*, but can be generated during the isolation of the liver and kidney forms. We routinely take our preparation through the CM-cellulose-chromatography step on the first day to avoid further generation of proteolytic forms. However, if we dialyse liver cytosol overnight and then continue with CM-Sephadex (C-50) chromatography, we do observe, in addition to the normal liver forms, a very prominent aa-homodimer, as previously reported (Hayes *et al.*, 1979, 1980). In addition, fractions

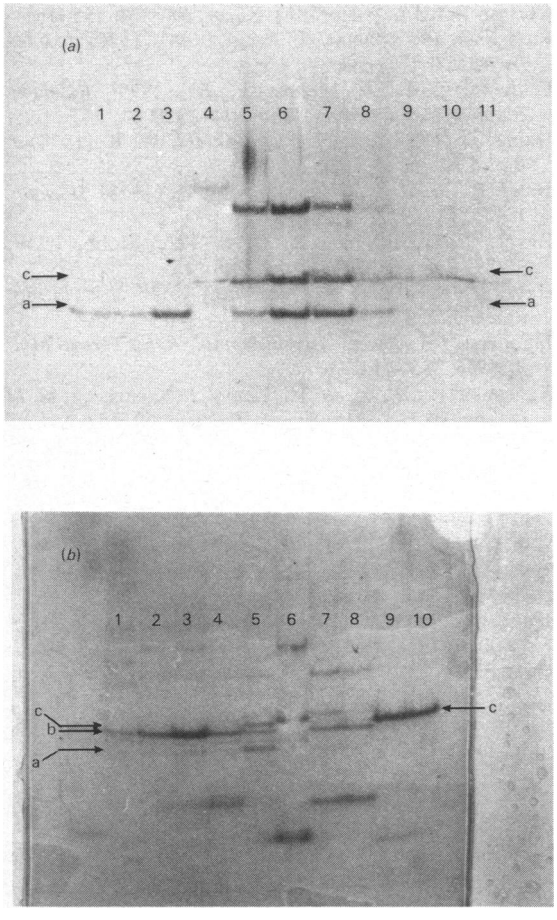


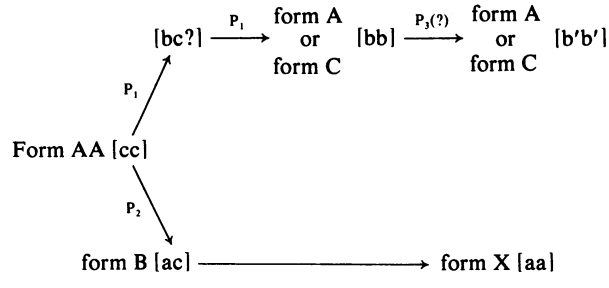
Fig. 2. *SDS/polyacrylamide-gel electrophoresis of fractions obtained after CM-cellulose chromatography of rat kidney cytosol (a) and rat testis cytosol (b) at pH6.7*
Electrophoresis was carried out by using a 15% (w/v) acrylamide resolving gel/3% acrylamide spacer gel run at 30mA for 6h. (*a*) The kidney fractions (see Fig. 1*a*) were run in tracks 1–11 as follows: fractions 8, 9 and 10, standards (ovalbumin, chymotrypsinogen A and cytochrome *c*), fractions 16, 17, 18, 19, 27, 29 and 31. (*b*) The testis fractions (see Fig. 1*b*) were run in tracks 1–10 as follows: fractions 10, 12, 13, 15, 17, standards (ovalbumin, chymotrypsinogen and cytochrome *c*), fractions 23, 25, 29 and 31.

eluting between forms B and C on CM-cellulose chromatography during our normal preparation of the liver forms have been analysed by SDS/polyacrylamide-gel electrophoresis, when we find no evidence for an aa-homodimer (N. C. Scully & T. J. Mantle, unpublished work). This suggests that the rapid gel filtration and DEAE-cellulose step may be removing proteinase contamination that is normally active only after homogenization (possibly lysosomal in origin), although the possibility of some covalent change in the protein other than proteolysis, e.g. the removal of carbohydrate, has not been ruled out.

CM-cellulose chromatography of lung and spleen cytosol revealed one major form, identified as glutathione *S*-transferase AA. This assignment was confirmed for both tissues by SDS/polyacrylamide-gel-electrophoresis, which showed only the c-subunit (results not shown).

An examination of the subunit composition of the kidney and testicular forms resolved by CM-cellulose chromatography at pH6.7 reveals an absence of the b-subunit in kidney and of the a-subunit in testis (see also Bass *et al.*, 1977; Bhargava *et al.*, 1980*b*). This observation is consistent with the absence of a proteinase (P_1) in kidney responsible for catalysing the c-subunit-to-b-subunit conversion and the deficiency of a proteinase (P_2) in testis responsible for the c-subunit-to-a-subunit conversion. Liver in this model possesses both proteinases P_1 and P_2, which appear to be absent in lung and spleen. The model for liver is shown in Scheme 1. It is noteworthy that this model postulates a direct c→a conversion. We have no evidence for significant amounts of a bc-heterodimer during CM-cellulose chromatography at pH6.7 or during fractionation of forms D and E from liver at pH6 (N. C. Scully & T. J. Mantle, unpublished work), so we conclude that it must be a transitory intermediate in the generation of forms A and C.

Although forms A and C are chromatographically separable, there is evidence for considerable sequence homology (Cornish-Bowden, 1978), and since these two forms are known to cross-react immunologically (Habig *et al.*, 1974; Guthenberg & Mannervik, 1979), we presume that one of the forms contains one or two modified b-subunits,



Scheme 1. *Postulated reaction pathway for the generation of the multiple forms of glutathione S-transferase in rat liver*
For details, see the text.

which we represent b′. Since subunits b and b′ are not separable by SDS/polyacrylamide-gel electrophoresis, b′ must arise as a result of very limited proteolysis (catalysed by P_3?) or some other post-translational modification, e.g. deamidation or phosphorylation. The model we are proposing has the merit that we can test the postulated product–precursor relationships experimentally by incubating various tissue homogenates with the purified forms of glutathione *S*-transferase radiolabelled with leucine and then monitoring radioactivity and glutathione *S*-transferase activity during CM-cellulose chromatography.

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