Identity of ligandin in rat testis and liver

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1. One of the main problems in the field of multifunctional proteins such as ligandin is the possibility that multiple forms and isoproteins may exist. Because liver ligandin [GSH (reduced glutathione) *S*-transferase B] consists of equal amounts of Ya (22 000 Da) and Yc (25 000 Da) subunits, and testis ligandin, prepared by the standard technique of anion-exchange and molecular-exclusion chromatography, contains more Yc subunit than Ya, it has been claimed that testis and liver ligandin are different entities. 2. We purified testis ligandin by immunoaffinity chromatography and have obtained a product identical with liver ligandin (Yc = Ya). This suggests that the differences previously described may be due to contamination of testis ligandin by a closely related species. In fact sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of testis GSH *S*-transferases separated by CM-cellulose chromatography showed that GSH *S*-transferase AA, present in large amounts, migrated in the same region as Yc subunit. 3. Testis ligandin prepared by the standard technique was similar to that reported [Bhargava, Ohmi, Listowsky & Arias (1980) *J. Biol. Chem.* **255**, 724–727] and contained more Yc subunit than Ya. CM-cellulose chromatography of this 'pure' preparation revealed significant amounts of GSH *S*-transferase AA migrating as Yc subunit, in addition to ligandin consisting of equal amounts of Ya and Yc subunits. 4. Our studies show that testis ligandin is identical with liver ligandin. Previously described differences are due to a contaminant identified as GSH *S*-transferase AA.

Ligandin is a basic protein (pI 8.9–9.3) present in abundance in the livers of all air-breathing vertebrates studied (Litwack *et al.*, 1971; Arias *et al.*, 1976). It has also been described in intestinal mucosa, proximal renal tubules and steroid-producing tissues of the rat (Fleischner *et al.*, 1972; Kirsch *et al.*, 1975; Bass *et al.*, 1977a). Rat liver ligandin has the chemical structure, enzymic activity, ability to bind non-substrate ligands and immunological properties of GSH *S*-transferase B (EC 2.5.1.18) (Habig *et al.*, 1974). SDS/polyacrylamide-gel electrophoresis of rat liver ligandin in a discontinuous system revealed two non-identical subunits, Ya (22 000 Da) and Yc (25 000 Da) (Bass *et al.*, 1977a). By using non-denaturing systems YaYa and YaYc dimers have been purified (Bass *et al.*, 1977a; Hayes *et al.*, 1979). It has been suggested that ligandin exists as a YaYc heterodimer (Listowsky *et al.*, 1976; Bhargava *et al.*, 1978) or as a mixture of a YaYa homodimer and a YaYc

heterodimer (Carne *et al.*, 1979). Indeed, Hayes *et al.* (1979) distinguish between ligandin (YaYa) and GSH *S*-transferase B (YaYc). However, this does not explain the finding by Bass *et al.* (1977a) and by Bhargava *et al.* (1980) that testis ligandin consists predominantly of a Yc fraction. We have investigated the possibility that a substance other than ligandin may contribute to this electrophoretic finding and to the differences in function attributed to testis ligandin. Our data suggest that GSH *S*-transferase AA accounts for part of the predominant Yc band seen on SDS/polyacrylamide-gel electrophoresis of testis preparations, that this protein is a major contaminant of testis ligandin purified by standard techniques, and that testis ligandin is made up of equal amounts of Ya and Yc subunits and is not different from liver ligandin.

Materials and methods

Chemicals

NADPH and bovine serum albumin were purchased from Miles Laboratories (Pty.) Ltd.,

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

Goodwood, South Africa. Glutathione reductase (Type IV) prepared from yeast was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cumene hydroperoxide (70% in cumene) was from Fluka A.G., Buchs, Switzerland. Complete Freund's Adjuvant and Agar Noble were from Difco Laboratories, Detroit, MI, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and SDS were from BDH Chemicals, Poole, Dorset, U.K. CNBr-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. Donkey anti-rabbit globulin was from Wellcome Reagents, Beckenham, Kent, U.K. ^{125}I was from The Radiochemical Centre, Amersham, Bucks., U.K. Dimethyl suberimide, 2HCl was from Pierce Chemical Co., Rockford, IL, U.S.A. All reagents used were of analytical grade.

Purification of ligandin

Ligandin was purified from the liver and testis of Long-Evans rats (250–300 g) as described by Kirsch *et al.* (1975) and Bhargava *et al.* (1980). In brief, cytosol prepared by centrifugation at 100 000 g for 120 min was obtained from 100 g of rat tissue, was dialysed against 0.01 M-Tris/HCl, pH 8.8 (buffer A), and chromatographed on a column of TEAE-cellulose (triethylaminoethyl-cellulose) in buffer A. The single protein peak eluted was pooled, concentrated (Amicon cell, PM-10 membrane) and chromatographed on a column of Sephadex G-100 in 0.01 M-sodium phosphate buffer (pH 7.4)/0.1 M-NaCl. Fractions exhibiting enzyme activity with 1-chloro-2,4-dinitrobenzene were pooled, concentrated, dialysed against buffer A and chromatographed on a column of QAE-Sephadex (quaternary aminoethyl-Sephadex) A-50 in buffer A. The single protein peak eluted was pooled and stored at 4°C.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 0.1% SDS was performed on vertical slab gels (Laemmli, 1970). The system comprised a 3% (w/v) stacking gel (3 cm × 14 cm × 0.2 cm) in 0.14 M-Tris/HCl buffer, pH 6.8, and a 5–25% (w/v) gradient gel (20 cm × 14 cm × 0.2 cm) in 0.56 M-Tris/HCl buffer, pH 8.8. Samples were prepared as described by Maizel (1971). Gels were fixed and stained for 2.5 h in 10% (v/v) trichloroacetic acid/1.0% Coomassie Brilliant Blue R in methanol/water (1:2, v/v) and destained in 5% methanol/7% acetic acid (both v/v).

Molecular weights were determined by comparison with standards of known molecular size.

Immunological studies

Antisera to liver and testis ligandin were raised by injecting 50 µg of purified proteins in Freund's complete adjuvant into popliteal lymph nodes of male albino rabbits. Thereafter, 50 µg of ligandin in adjuvant was injected into multiple subcutaneous

sites at 3-week intervals. Animals were bled 10 days after booster inoculations. The separated sera were tested for specific antibody production by immunodiffusion and immunoelectrophoresis (Bass *et al.*, 1977a). Radial immunodiffusion used 1.2% agar gels (Ouchterlony, 1958) and immunoelectrophoresis used 1% agar gels (Bass *et al.*, 1977a). Gels were thoroughly washed with iso-osmotic saline (0.9% NaCl) before staining with Amido Black. Affinity reagents, consisting of the globulin fraction either of anti-(rat liver ligandin) serum or anti-(rat testis ligandin) serum prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Hebert *et al.*, 1973), were coupled overnight at 4°C to CNBr-activated Sepharose 4B. The remaining active groups were treated with 1 M-ethanolamine at pH 8 for 2 h, after which several washing cycles (0.1 M-acetate buffer/1 M-NaCl, pH 8.0, and 0.1 M-borate buffer/1 M-NaCl, pH 4.0) were carried out to remove non-covalently adsorbed protein. Thus 98.4% of liver antibody and 94.2% of testis antibody were coupled. Partially purified rat tissue ligandin (TEAE-cellulose eluate, Fig. 3) was applied to a column (10 cm × 0.9 cm) of affinity material. After unbound protein had been washed from the column, immunoreactive material was eluted with 0.1 M-glycine, pH 3.0. The pH of all fractions was immediately adjusted to 7.4 with 1 M-Tris buffer.

Radioimmunoassay of ligandin from rat tissues was performed by the double antibody method of Morgan & Lazarow (1963), with anti-(rat liver ligandin) serum as the first antibody. The assay procedure and the antisera used in this study have been described in detail (Bass *et al.*, 1977b).

Separation of glutathione S-transferases

Rat liver and testis supernatants were chromatographed on a DEAE-cellulose column (2.6 cm × 35 cm) in 0.01 M-Tris/HCl buffer, pH 8.0. Fractions exhibiting absorbance at 280 nm were pooled and concentrated to a volume of 10 ml (Amicon cell, PM-10 membrane). After dialysis into 0.01 M-potassium phosphate buffer, pH 6.7 (buffer C), the preparation was applied to a CM-cellulose column (2.6 cm × 30 cm) previously equilibrated with buffer C (Habig *et al.*, 1974). A 600 ml linear gradient of 0–120 mM-HCl in buffer C was applied to the column, and 3 ml fractions were collected and stored at 4°C before assay.

In a separate experiment, testis ligandin prepared as described by Bhargava *et al.* (1980) was chromatographed on a CM-cellulose column (1.6 cm × 10 cm) as described above.

Assays

Glutathione S-transferase activity was measured by following the conjugation of GSH with either 1-chloro-2,4-dinitrobenzene at 340 nm or 1,2-dichloro-4-nitrobenzene at 345 nm (Habig *et al.*,

1974). Glutathione peroxidase activity was assayed, as described by Prohaska & Ganther (1977), with 0.1mM-cumene hydroperoxide dissolved in 10% (v/v) ethanol. One enzyme unit is defined as 1 μ mol of NADPH oxidized/min. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Covalent cross-linking of ligandin

Ligandin in 0.05 M-triethanolamine buffer (pH 8.0)/0.05 M-KCl/0.4 M-NaCl was mixed with dimethyl suberimidate at 4°C to give final concentrations of 0.3 mg of protein/ml and 30 mg of cross-linking reagent/ml. The reaction was terminated by addition of an equal volume of 0.3 M-ethanolamine, before electrophoresis in SDS on polyacrylamide gradient gels (Davies & Stark, 1970).

Results

Purification of testis ligandin

Specific activity, with 1-chloro-2,4-dinitrobenzene and GSH as substrates, was determined at each stage of testis ligandin purification by the method of Kirsch *et al.* (1975) and Bhargava *et al.* (1980). The mean specific activity of the final product of four separate testis preparations was 15.6 μ mol/min per mg of protein. SDS/polyacrylamide-gel electrophoresis of each stage of purification is shown in Fig. 1. The final preparation of testis ligandin has more Yc subunit than Ya. These findings and those of difference spectroscopy (results not shown) are identical with those obtained by Bhargava *et al.* (1980), and suggest that we are dealing with the same preparation.

Antisera

Radial immunodiffusion and immunoelectrophoresis of anti-(liver YaYa ligandin) or anti-(liver YaYc ligandin) against liver and testis cytosol revealed single lines of identity. Antisera raised against testis 'ligandin' revealed a single line of identity when immunoelectrophoresed against testis cytosol. However, a faint second line was noticed on immunoelectrophoresis against liver cytosol (Fig. 2).

Affinity chromatography

The profile obtained when the testis protein peak (TEAE-cellulose eluate) was applied to an anti-(liver ligandin) affinity column is shown in Fig. 3. Fractions were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 4). The TEAE-cellulose eluate had more Yc subunit than Ya. In contrast, the material recognized by the matrix-coupled liver ligandin antibody (elution volume 85 ml) had equal quantities of the two subunits. Unbound fractions (elution volume 15 ml) contained no Ya subunit, but

Fig. 1. SDS/polyacrylamide (5–25%, w/v)-gradient-gel electrophoresis at each stage of testis ligandin purification

(1) Molecular-weight markers (94 000, 67 000, 43 000, 30 000, 20 100, 14 400 daltons). (2) Testis cytosol (100 000 g supernatant). (3) Protein peak after TEAE-cellulose chromatography. (4) Peak exhibiting GSH-chlorodinitrobenzene-conjugating activity after Sephadex G-100 chromatography. (5) Pooled fractions from QAE-Sephadex A-50 eluate. Between 10 and 30 μ g of protein was loaded per sample. Samples were prepared as described by Maizel (1971).

Fig. 2. Immunoelectrophoresis of testis ligandin antibody against liver and testis cytosols in 1% agar gel

The peripheral wells contain liver cytosol and testis cytosol (CYT; 1 μ g in 10 μ l). The central trough contains 10 μ l of rabbit antiserum (AB) to testis ligandin. Electrophoresis was performed in 0.07 M-veronal buffer, pH 8.7, at 12 mA for 2 h. The precipitates were stained with Amido Black.

Fig. 3. Chromatography of partially purified (TEAE-cellulose eluate) rat testis ligandin on anti-ligandin-Sepharose 4B affinity columns

The elution profiles for anti-(liver ligandin) and anti-(testis ligandin) affinity columns are identical. Unbound material was eluted in the first peak (elution volume 10–40 ml). Bound material was eluted with 0.1M-glycine, pH 3.0 (elution volume 80–95 ml).

Fig. 4. SDS/polyacrylamide-gel electrophoresis of peaks eluted after anti-(liver ligandin)-Sepharose 4B affinity chromatography

(1) Testis TEAE-cellulose eluate (starting material); (2) material at elution volume 15 ml; (3) material at elution volume 30 ml; (4) material after elution with 0.1M-glycine, pH 3.0. See Fig. 3 for details.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of eluate from anti-(testis ligandin)-Sepharose 4B affinity column

(1) Testis TEAE-cellulose eluate (starting material), (2) material eluted at 15 ml, (3) material eluted at 25 ml, (4) material eluted after application of 0.1M-glycine buffer, pH 3.0 (elution volume 80–95 ml). See Fig. 3 for details.

later fractions (elution volume 30 ml) contained trace amounts of this species.

When the same testis starting preparation was applied to a column of anti-(testis ligandin) affinity material, SDS/polyacrylamide-gel electrophoresis (Fig. 5) revealed more Yc subunit than Ya in the immunoreactive peak (elution volume 85 ml).

Elution of testis GSH S-transferases from CM-cellulose

CM-cellulose chromatography resolved liver DEAE-cellulose eluate into five peaks of GSH S-transferase activity (GSH-chloronitrobenzene conjugating activity) similar to those described by Habig *et al.* (1976). Fig. 6 shows the results of a similar experiment with testis DEAE-cellulose

Fig. 6. Elution pattern from CM-cellulose of testis DEAE-cellulose eluate at pH 6.7

GSH S-transferase activity was assayed with 1-chloro-2,4-dinitrobenzene (CDNB, ○) and 1,2-dichloro-4-nitrobenzene (DCNB, ●). Enzyme activity is expressed as $\mu\text{mol}/\text{min}$ per ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene HOOH) as substrate (■). Results were expressed as μmol of cumene hydroperoxide/min, by using the absorption coefficient (ϵ) for NADPH of $6 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Ligandin as measured by anti-(liver ligandin) radioimmunoassay is expressed as ng of ligandin/tube (Δ). The KCl gradient was started after peak 1 had been eluted (elution volume 0–180 ml).

Fig. 7. CM-cellulose chromatography of 'pure' testis ligandin at pH 6.7

The KCl gradient (—, 0–120 mm) was started at the beginning of the column run. GSH S-transferase activity was assayed with chlorodinitrobenzene (CDNB, ○) and expressed as $\mu\text{mol}/\text{min}$ per ml. GSH peroxidase II activity was assayed with cumene hydroperoxide (cumene HOOH) and expressed as $\mu\text{mol}/\text{min}$ (■). Radioimmunoassay with anti-(liver ligandin) measured ng of ligandin per tube (Δ). Comparisons of the standard preparation of testis ligandin (1) with fractions from each peak (2 and 3) by SDS/polyacrylamide-gel electrophoresis are shown.

eluate. Peak 1 (elution volume 40–70 ml) represents material that did not bind to the column at pH 6.7. After initiation of the salt gradient, a further four peaks were obtained, peak 2 (elution volume 460–530 ml), peak 3 (530–600 ml), peak 4 (600–660 ml) and peak 5 (690–780 ml). Dichloronitrobenzene-conjugating activity was limited to peaks 2 and 4. GSH peroxidase II activity with cumene hydroperoxide as substrate was present in peak 5 and to a lesser extent in peaks 2 and 4. GSH

peroxidase I activity was not measured in this experiment. Immunoreactivity determined by radioimmunoassay with antibody raised against liver ligandin was present in fractions eluted between 480 and 600 ml. No immunoreactive material was found in peak 5. SDS/polyacrylamide-gel electrophoresis of the immunoreactive peak revealed equal amounts of Ya and Yc subunits, but for peak 5 it revealed a major band in the Yc region but no Ya subunit.

Chromatography of 'purified' testis ligandin

Fig. 8. SDS/polyacrylamide-gel electrophoresis of 'pure' testis ligandin cross-linked with dimethyl suberimidate (1) Testis ligandin, purified by standard procedures, no dimethyl suberimidate added. (2) Testis ligandin, after addition of dimethyl suberimidate and cross-linking reaction allowed to proceed for 1.2 h under conditions described in the Materials and methods section, (3) after cross-linking for 2.5 h, and (4) after cross-linking for 3.5 h.

yielded two peaks of GSH-chlorodinitrobenzene-conjugating activity. Both peaks had GSH peroxidase II activity. Only peak 1 contained immunoreactive material as determined by radioimmunoassay by using antiserum raised against liver ligandin. SDS/polyacrylamide-gel electrophoresis of peak-1 material revealed equal amounts of Ya and Yc subunits; peak 2 consisted entirely of Yc subunit (Fig. 7).

Covalent cross-linking of testis ligandin

SDS/polyacrylamide-gel-electrophoretic analysis of covalently cross-linked testis ligandin resulted in two bands in the region of approx. 50 000 mol.wt. after cross-linking had been allowed to proceed for 2.5 h under the conditions described in the Materials and methods section (Fig. 8).

Discussion

Liver ligandin, as defined by Bass *et al.* (1977a), consists of equal amounts of Ya and Yc subunits. In contrast, the Yc subunit accounts for as much as 90% of testis ligandin (Bhargava *et al.*, 1980; Bass *et al.*, 1977a). By the standard purification procedure we were able to purify a protein with subunit structure, amino acid composition, binding characteristics (results not shown) and GSH *S*-transferase activity similar to that described by Bhargava *et al.* (1980). They found testis ligandin to have a lower affinity than liver ligandin for bilirubin and for sulphobromophthalein. Circular-dichroism spectra

revealed the absence of the primary binding site for bilirubin (Bhargava *et al.*, 1980).

When testis ligandin was prepared by affinity chromatography, with antibody raised against liver ligandin, it consisted of equal amounts of Ya and Yc subunits. This was clearly different from testis ligandin preparations obtained by the standard purification procedure. The testis starting material contained more Yc than Ya subunit (Fig. 5, lane 1). However, a considerable amount of Yc subunit was eluted in the wash (Fig. 5, lanes 2 and 3), suggesting that this may not have been recognized by the antibody. The immunoreactive material eluted with glycine buffer, pH 3.0, consisted of equal amounts of Yc and Ya subunits (Fig. 5, lane 4), resembling the proportions of these subunits found in liver ligandin. In addition, antibodies raised against testis ligandin prepared by standard methods consistently failed to meet immunodiffusion and immunoelectrophoretic criteria for purity when tested against liver cytosol. Furthermore, SDS/polyacrylamide-gel electrophoresis of covalently cross-linked testis ligandin revealed more than one band in the 50 000 Da region. This finding differed from that reported by Bhargava *et al.* (1978) for liver ligandin and thus may suggest the presence of another protein in our testis preparations. Our suspicion that standard preparations of testis ligandin could be contaminated by a closely related protein were strengthened by the findings of Hayes *et al.* (1980) and Scully & Mantle (1980), who studied the subunit composition of liver GSH *S*-transferases. The GSH *S*-transferases of rat liver (AA, A, B, C, D and E) have been classified according to their reverse order of elution from CM-cellulose (Habig *et al.*, 1976). Transferases D and E are eluted before the salt gradient is applied, after which transferases C, B, A and AA are eluted sequentially. Although there is overlap of substrate specificity, the various transferases may be identified by their order of elution from CM-cellulose and their ability to conjugate GSH to various substrates. All conjugate chlorodinitrobenzene. Only transferases A and C conjugate GSH to dichloronitrobenzene (Habig *et al.*, 1974), and transferases B, A and AA have GSH peroxidase activity (Prohaska & Ganther, 1977; Lawrence *et al.*, 1978; Irwin *et al.*, 1980). On SDS/polyacrylamide-gel electrophoresis, liver transferase AA migrates as subunit Yc, liver transferase B migrated as YaYc dimer, and transferases A and C [which show immunological identity (Habig *et al.*, 1974)] migrate as Yb subunit. This finding led Hayes *et al.* (1979) to differentiate between GSH *S*-transferase B (YaYc) and ligandin (YaYa). Since our study has been aimed at determining the subunit composition of a protein in testis which reacts immunologically with anti-(liver ligandin), the term ligandin has been used throughout, although in the

light of our findings the term GSH *S*-transferase B may be more appropriate.

To determine whether testis transferase AA migrated as Yc subunit, we separated the testis GSH *S*-transferases by CM-cellulose chromatography and used order of elution, SDS/polyacrylamide-gel electrophoresis, radioimmunoassay and catalytic activity with different substrates to characterize the various fractions. Activity with chlorodinitrobenzene and dichloronitrobenzene resembled that seen in liver. We found GSH peroxidase activity in peaks corresponding to liver GSH *S*-transferases B, A and AA. Of interest is the fact that transferase AA accounts for most of the GSH peroxidase II activity in testis cytosol. SDS/polyacrylamide-gel electrophoresis of the CM-cellulose peak with the elution characteristics of transferase AA revealed a major band in the Yc-subunit region, similar to findings reported by Scully & Mantle (1981). This peak was not recognized by antibody raised against liver ligandin. Immunoreactivity was confined to fractions eluted in the transferase-B region of the CM-cellulose profile. On SDS/polyacrylamide-gel electrophoresis, this peak consisted of equal amounts of Ya and Yc subunits.

It was apparent that if GSH *S*-transferase AA was present in standard preparations of ligandin it would not be detected on SDS/polyacrylamide-gel electrophoresis. We thus used CM-cellulose chromatography to study 'pure' testis ligandin and found it to consist of GSH *S*-transferase B (consisting of equal amounts of Ya and Yc subunits and recognized by antibody raised against the liver Ya subunit) and GSH *S*-transferase AA (which resembles Yc subunit on SDS/polyacrylamide-gel electrophoresis). Our finding that standard preparations of testis ligandin are contaminated by GSH *S*-transferase AA offers a new explanation for studies previously reported and interpreted differently.

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