A study of the structures of the YaYa and YaYc glutathione *S*-transferases from rat liver cytosol

Evidence that the Ya monomer is responsible for lithocholate-binding activity

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The two dimeric lithocholic acid-binding proteins previously identified as ligandin (YaYa) and glutathione *S*-transferase B (YaYc) were isolated from rat liver cytosol. These proteins have molecular weights of 44 000 and 47 000 respectively. The recovery of these two proteins from liver was not affected by the addition of the proteinase inhibitor Trasylol. No spontaneous interconversion between these two proteins was observed on storage. YaYa and YaYc proteins yielded peptides of identical molecular weight after limited digestion with *Staphylococcus aureus* V8 proteinase. Analytical and preparative tryptic-digest peptide 'maps' showed that all the soluble peptides obtained from YaYa protein were also recovered from YaYc protein. Approximately six extra soluble peptides, which were not recovered from YaYa protein, were obtained from the tryptic digest of YaYc protein. Subdigests of the insoluble tryptic-digest 'cores' also resulted in the recovery of identical peptides from both proteins. Evidence is presented that the Ya subunit possessed by both proteins is identical; glutathione *S*-transferase B is a hybrid of ligandin and glutathione *S*-transferase AA. The Ya monomer is responsible for lithocholate binding.

Three groups of workers independently isolated, from rat liver cytosol, anion-binding proteins that were each dimeric, basic (pI 8.4–9.0) and possessed a molecular weight of about 45 000. They were shown to be immunochemically similar and demonstrated similar affinities for a variety of ligands. The proteins were concluded to be identical and the term ligandin was adopted to describe them (Litwack *et al.*, 1971).

Ligandin was subsequently reported to be identical with glutathione *S*-transferase B (Habig *et al.*, 1974a), one of at least seven enzymes having glutathione *S*-transferase activity in rat liver cytosol (Jakoby *et al.*, 1976). The enzymes each comprise two of at least three monomers that were identified by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; these are monomers Ya (mol.wt. 22 000), Yb (mol.wt. 23 500) and Yc (mol.wt. 25 000) (Bass *et al.*, 1977; Hayes *et al.*, 1980).

Although the terms ligandin and glutathione *S*-

transferase B were thought to describe a single protein, there is now doubt as to whether the three initial preparations of ligandin actually comprised only one protein. Bass *et al.* (1977) showed that ligandin from rat liver comprised predominantly Ya monomers, but Daniel *et al.* (1977) and Bhargava *et al.* (1978) have shown that their preparations of hepatic ligandin comprise equal amounts of Ya and Yc monomers. However, Carne *et al.* (1979) have resolved their ligandin preparation into two proteins, which comprise YaYa and YaYc dimers. We have reported that ligandin (prepared by the method of Arias *et al.*, 1976) is composed of Ya subunits and that glutathione *S*-transferase B comprises Ya and Yc subunits (Hayes *et al.*, 1979).

The relationship between YaYa and YaYc proteins is not clear, for two reasons. Firstly, the Ya subunits, which both proteins contain, has only been identified by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and it is not known whether this subunit is identical in both proteins. The Ya subunits may exist in ionically distinct forms in a situation analogous to glutathione *S*-transferases A and C, both of which are Yb homodimers that are completely resolved by CM-Sephadex chromato-

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graphy (Hayes *et al.*, 1980). Secondly, it is not known whether the Yc monomer is coded for by the same gene(s) that code(s) for the Ya subunit(s). The proteins may be coded separately, and represent true isoenzymes, or variants that arise from post-translational modifications of a single parental polypeptide.

No definitive data have been published that enable these possibilities to be distinguished. Certain workers have reported that the Yc monomer of YaYc protein is converted during storage into a monomer that is electrophoretically identical with monomer Ya (Listowsky *et al.*, 1976; Daniel *et al.*, 1977). Not only is it unclear whether this 'YaYa protein' produced during storage is identical with native YaYa protein, but this storage phenomenon is controversial and has not been reproduced by all workers (Bass *et al.*, 1977). Habig *et al.* (1974b) and Bhargava *et al.* (1978) have also investigated the possibility that proteolytic degradation *in vitro* is responsible for the formation of different transferases. Habig *et al.* (1974b) found no alteration in the yield of the different transferases when the serine proteinase inhibitor phenylmethanesulphonyl fluoride was included during their purification procedures. Bhargava *et al.* (1978) also found no alteration in the recovery of YaYc protein when they included phenylmethanesulphonyl fluoride in their purification procedure. Furthermore, they found no preferential loss of the Yc monomer from YaYc protein when it was incubated with crude rat liver homogenate under conditions that favoured proteolysis. Neither group of workers investigated possible proteolysis of transferase AA (YcYc protein). However, Scully & Mantle (1981) have proposed that all the transferases are generated by proteolysis of YcYc protein. In particular, these workers reported that YaYa protein is recovered only after extensive dialysis of whole cytosol. They proposed that it is formed as the result of proteolysis *in vitro*.

Ketterer and his colleagues have studied YaYa and YaYc proteins. They reported that these proteins possess similar enzyme activities, amino acid compositions and peptide 'maps' (Ketterer *et al.*, 1976; Carne *et al.*, 1979). However, Strange *et al.* (1977) have reported that lithocholate inhibits the enzyme activity of YaYa protein but not YaYc protein, indicating that the transferase activity of these two enzymes can be distinguished. Further, Hayes *et al.* (1979) showed that the peptide 'maps' of these two proteins differed, after limited chymotrypsin digestion, suggesting that there are differences in the primary structure of these two proteins.

We now describe a structural comparison of YaYa and YaYc proteins. The effect of Trasylol (a proteinase inhibitor) on the yield of these two lithocholate-binding proteins was investigated. The effects of α -mannosidase, *N*-acetylglucosaminidase

and neuraminidase on their subunit compositions were examined to determine whether glycosylation could account for their different electrophoretic mobilities. Analytical and preparative peptide 'maps' were constructed to compare the primary structures of YaYa and YaYc proteins. The possibility that the Ya subunit in both proteins is identical was investigated by analysis of the products formed after urea denaturation of YaYc protein. The lithocholate-binding activities of the Ya and Yc subunits were examined.

Materials and methods

Chemicals

The chemicals used were readily available commercially and were of analytical grade.

Enzymes

Neuraminidase, α -mannosidase, β -*N*-acetylglucosaminidase and α_1 -antitrypsin were from Sigma Chemical Co., Poole, Dorset, U.K. *Staphylococcus aureus* V8 proteinase and trypsin were a gift from Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, Scotland, U.K.

Buffers

The composition of the buffers used and the temperatures at which they were prepared were: buffer A, 10mM-sodium phosphate buffer, pH 7.4 (20°C); buffer B, 100mM-sodium phosphate buffer, pH 6.5 (20°C).

Purification of YaYa and YaYc proteins

YaYa and YaYc proteins were purified from perfused rat livers essentially as described by Strange *et al.* (1977). Modifications were made to this procedure to scale-up the purification. These have been described (Hayes, 1980).

Discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed to determine the subunit compositions and purity of transferase-containing fractions as previously described (Hayes *et al.*, 1979).

Analysis of lithocholate-binding by whole cytosol

To minimize possible proteolytic degradation of the glutathione *S*-transferases, cytosol was prepared in the presence of the general proteinase inhibitor Trasylol (Trautschold *et al.*, 1966) and was applied immediately to CM-Sephadex to separate the lithocholate-binding proteins. Cytosol from two male rats (250g) was prepared in the presence of Trasylol (100 kallikrein-inhibitor units/ml) in buffer A containing 250mM-sucrose (Strange *et al.*, 1976). The

cytosol was diluted 6-fold with buffer A. To a portion of this solution (30 ml, 150 mg of protein) [^{14}C]lithocholic acid (1 μCi , 18 nmol) was added and the mixture was applied to a column (2.2 cm $\times$ 15 cm) of CM-Sephadex without dialysis, as described by Strange *et al.* (1977). A NaCl gradient of 0–80 mM and a 100 ml mixing reservoir were used. Fractions of volume 3.2 ml were collected, and the enzyme activity (with either 1-chloro-2,4-dinitrobenzene or 1,2-dichloro-4-nitrobenzene as substrate), Na^+ concentration, radioactivity and subunit compositions were determined.

Enzymic removal of carbohydrate from YaYa and YaYc proteins

Portions of YaYa and YaYc proteins (80 μg of protein) were incubated in buffer B (25°C; 48 h) with a mixture of α -mannosidase (10 μg of protein), *N*-acetylglucosaminidase (5 μg of protein) and neuraminidase (10 μg of protein). The digestion of α_1 -antitrypsin (80 μg of protein) served as a control. The digests were terminated by heating (95°C, 10 min) after addition of Bromophenol Blue, 2-mercaptoethanol, sodium dodecyl sulphate and sucrose to the mixtures to give final concentrations of 0.002%, 1%, 4% and 10% respectively. Portions (30 μl) of the digests, containing 15 μg of YaYa or YaYc protein or α_1 -antitrypsin, were analysed by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a 11.5% resolving gel.

Peptide 'mapping' by limited proteolytic digestion in the presence of sodium dodecyl sulphate

The method used was that described by Cleveland *et al.* (1977). Portions of YaYa and YaYc proteins (50 μg of protein) were heated in the presence of 0.2% (w/v) sodium dodecyl sulphate (95°C, 2 min), cooled (25°C, 5 min) and then incubated (37°C, 45 min) with various amounts of *Staphylococcus aureus* V8 proteinase (14, 1.4, 0.14, 0.014 or 0.0014 μg of protein), and the digests were analysed by discontinuous sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described by Hayes *et al.* (1979).

Total amino acid compositions

These were determined as described by Ambler & Brown (1967). No corrections were made for the destruction of serine and threonine.

N-Terminal analysis

The dansylation, subsequent hydrolysis and separation of dansyl-amino acids on polyamide-coated sheets were performed as described by Thatcher (1975).

High-voltage paper electrophoresis

This was performed at pH 6.5, 3.5 and 2.1 in

Michl electrophoresis tanks (Ambler, 1963). Whatman 3MM paper was used for electrophoresis at pH 6.5 and 3.5 and Whatman no. 1 paper was used for electrophoresis at pH 2.1.

Descending paper chromatography

This was performed with Whatman 3MM paper, with butan-1-ol / acetic acid / water / pyridine (15:3:12:10, by vol.) as solvent.

Analytical peptide 'mapping' after total proteolysis

Portions of YaYa and YaYc proteins (approx. 1.5 mg of protein) were oxidized (60 min) with 0.2 ml of performic acid (Hirs, 1967), and the reaction products were desiccated. Digestion of the two proteins was performed at 37°C for 4.5 h with a substrate/trypsin ratio of 50:1 (w/w) (Bhargava *et al.*, 1978). The reaction was terminated by freeze-drying. After dissolution of the freeze-dried digestion products in 100 μl of 0.1 M- NH_3 , the peptides from YaYa protein and YaYc protein were applied to Whatman 3MM paper and separated in the first dimension into 'basic' peptides, 'neutral' peptides and 'acidic' peptides by high-voltage paper electrophoresis at pH 6.5. The 'basic' and the 'acidic' peptides were subjected to electrophoresis at pH 3.5 in the second dimension to yield a two-dimensional 'map'. The 'neutral' peptides were further fractionated in the first dimension by electrophoresis at pH 3.5 and separated in the second dimension by descending chromatography. The peptides were located by dipping the paper in 0.2% ninhydrin solution in acetone containing 3% (v/v) collidine. The construction of the basic, acidic and neutral peptide 'maps' and the nomenclature used to define these peptides has been described (Hayes, 1980).

Isolation and analysis of tryptic-digest peptides

Portions of oxidized YaYa and YaYc proteins (25 mg of protein) were digested with trypsin as described above. The freeze-dried digests were dissolved in 0.2 ml of 50% (v/v) formic acid, and the solutions were then diluted to 2.0 ml with distilled water before chromatography. The insoluble material ('core') was removed by centrifugation (at 4000 g for 10 min), and the supernatant was applied to Sephadex G-25 equilibrated and eluted with 5% (v/v) formic acid. Three columns (each 90 cm $\times$ 1 cm) in series were used. The flow rate was 10 ml/h and 2 ml fractions were collected. A portion (200 μl) of each column fraction was desiccated *in vacuo* over NaOH. The dried material was then redissolved in 25 μl of 50% (v/v) pyridine and subjected to electrophoresis (at 3000 V for 45 min) at pH 6.5. The resulting peptide 'map' was used to decide which fractions to combine to facilitate the subsequent preparative electrophoretic separation at pH 6.5. Peptides were isolated by electrophoresis at pH 6.5, followed by descending paper chromatography and a final paper

electrophoresis at pH 3.5. Between each of these steps the peptides were eluted from the paper with 0.1M-NH₃ and their purity was assessed by *N*-terminal amino acid analysis. The purified peptides were analysed by hydrolysis in 6M-HCl followed by electrophoresis at pH 2.1.

Subdigests of tryptic 'core' material

The insoluble material (in 5% formic acid) removed from the tryptic digests of YaYa and YaYc proteins by centrifugation was redissolved in 2 ml of 50% formic acid. Portions (0.1 mg, 50 µl) of the redissolved 'cores' were desiccated. The dried material was resuspended in 0.2 ml of 200 mM-ammonium acetate buffer, pH 9.0, and 20 µg of trypsin, V8 proteinase or chymotrypsin (substrate/ proteolytic enzyme ratio 5:1, w/w) were added. The digests were incubated at 37°C for 6 h before they were desiccated. The dried material was redissolved in 20 µl of 0.1M-NH₃ and subjected to electrophoresis at pH 6.5 as described above.

Formation of Ya and Yc homodimers from YaYc protein

The subunits from YaYc protein were separated by urea denaturation, and the products formed after rehybridization were identified by CM-Sephadex chromatography. A solution (35 ml) of 8M-urea in buffer B containing 1 mM-EDTA and 1 mM-reduced glutathione was added to a solution of purified YaYc protein (5 ml, about 5 mg of protein) in buffer A containing 1 mM-EDTA and 1 mM-reduced glutathione. The denaturation was allowed to proceed (at 20°C) for 1 h, after which the urea was removed by dialysis (18 h, 4°C) against three changes, each of 2 litres, of buffer A. Precipitated material was removed by centrifugation (at 10000 g for 10 min at 4°C) and analysed by polyacrylamide-gel electrophoresis. To enable the products to be identified the supernatant was applied to a CM-Sephadex column (2.2 cm × 13 cm). CM-Sephadex chromatography was performed with a 50 ml mixing reservoir and a 0–80 mM-NaCl gradient, and the eluate was analysed as described above.

Analysis of lithocholate binding by Ya and Yc subunits

Portions of YaYa, YaYc or YcYc protein (2 ml, about 0.3 mg of protein), obtained from CM-Sephadex chromatography of urea-treated YaYc protein, were incubated (at 4°C for 30 min) with [¹⁴C]-lithocholic acid (0.5 µCi, 8 nmol). The protein/lithocholic acid mixture was applied to a Sephadex G-75 column (2.2 cm × 19 cm), which was eluted (at 16 ml/h at 4°C) with 20 mM-sodium phosphate/100 mM-NaCl buffer, pH 7.4. The void volume was 30 ml and the salt volume was 70 ml. Fractions of

volume 2.7 ml were collected and the enzyme activity and radioactivity were determined.

Results

Lithocholate binding by whole cytosol

To minimize proteolysis, cytosol was prepared at 4°C in the presence of Trasylol and was applied to CM-Sephadex within 2 h of removal of the livers. Fig. 1 shows the elution of the transferases from CM-Sephadex. The peaks were numbered 1–5, as previously described (Hayes *et al.*, 1980). The resolution of peaks 1 and 2 was compromised because the volume of sample applied was greater than previously used. This was necessitated as the cytosol had to be diluted before chromatography to lower the ionic strength of the sample. The elution profile of the transferases eluted with the salt gradient is similar to that obtained from dialysed cytosol prepared in the absence of Trasylol (Hayes *et al.*, 1979). In particular, the recovery of YaYa protein (peak 3) was not diminished by omitting the dialysis step we previously used.

Stability of YaYa and YaYc proteins during storage

YaYa and YaYc proteins were examined at monthly intervals during this study by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis to determine whether these proteins are interconvertible during storage. No change in the composition of the proteins was observed after storage for 9 months (–10°C) in either 50 mM-ammonium acetate buffer, pH 8.3, or buffer A, pH 7.4. The addition of 1 mM-reduced glutathione and 1 mM-EDTA to these proteins also failed to alter the subunit compositions of YaYa and YaYc proteins.

Amino acid compositions

No significant difference between the compositions of the two proteins was observed. No glucosamine or galactosamine was recovered from either protein.

Carbohydrate-removing digests

To investigate the possibility that the differences in subunit composition of the two proteins resulted from glycosylation, the proteins were incubated with neuraminidase, α-mannosidase and *N*-acetylglucosaminidase. The subunit compositions of YaYa and YaYc proteins were unchanged and there was no evidence of the Yc monomer being converted into the Ya monomer by the action of these enzymes. The molecular weight of the control, α₁-antitrypsin, was decreased from about 50 000 to about 45 000 during digestion.

Fig. 1. Elution pattern of cytosol and [¹⁴C]lithocholic acid from CM-Sephadex

Fresh cytosol (approx. 150 mg of protein) was diluted 6-fold with buffer A and added to CM-Sephadex, after the addition of [¹⁴C]lithocholic acid, as described in the text. Fractions (3.2 ml) were collected and the radioactivity (●) and Na⁺ concentrations (■) were determined (a). Glutathione *S*-transferase activity was also measured with 1-chloro-2,4-dinitrobenzene (▲) or 1,2-dichloro-4-nitrobenzene (○) as substrate (b).

'Cleveland gels' of YaYa and YaYc proteins

A comparison between the structures of the two proteins was initially performed by using 'Cleveland gels' after partial digestion with *Staphylococcus aureus* V8 proteinase. Electrophoresis revealed that both proteins were equally susceptible to digestion and that three major digestion products (mol.wts. about 9000, 12000 and 15000) were common to both proteins (Fig. 2).

N-Terminal analysis

After dansylation of YaYa and YaYc proteins, no N-terminal residues were detected from either protein. Both proteins appear to possess blocked N-termini. This is unlikely to be due to steric hindrance, as both proteins were oxidized with performic acid before dansylation.

Analytical peptide 'maps'

Fig. 3 shows the two-dimensional 'map' of the acidic peptides from YaYa and YaYc proteins. This shows that at least six peptides (A1a–A6a and A1b–A6b) are common to both proteins. YaYc protein also appeared to contain two peptides (A7b and A8b) that were not recovered from YaYa protein.

Fig. 4 shows the two-dimensional 'map' of the basic peptides from the two proteins. This shows that 16 peptides (B1a–B16a and B1b–B16b) appear to be common to both proteins and that the main bulk of the peptides ran diagonally across the electrophoresis paper forming a V-shaped 'map'. Differences between YaYa and YaYc proteins were observed in the peptides outside this main V-shape. YaYc protein yielded an additional peptide (B17b)

Fig. 2. *Partial digestion of YaYa and YaYc proteins with Staphylococcus aureus V8 proteinase*
Portions (50 μ g) of YaYa and YaYc proteins were digested with various amounts of *Staphylococcus aureus* V8 proteinase as described in the text. After 45 min the digestion products were applied to a sodium dodecyl sulphate/polyacrylamide gel and run from the cathode (top) to the anode (bottom). The samples applied were: 1, V8 proteinase (7 μ g); 2–6, YaYa protein (25 μ g) and 0.0007 μ g, 0.007 μ g, 0.07 μ g, 0.7 μ g and 7 μ g of V8 proteinase respectively; 7–11, YaYc protein (25 μ g) and 7 μ g, 0.7 μ g, 0.07 μ g, 0.007 μ g and 0.0007 μ g of V8 proteinase respectively; 12, V8 proteinase (7 μ g).

that was not found in YaYa protein. This was clearly resolved by using electrophoresis at pH 6.5 and pH 3.5. An additional peptide (B18b) in the YaYc-protein digest that migrated between peptides B12b and B13b was not observed between peptides B12a and B13a in the YaYa-protein digest.

Fig. 5 shows the 'map' of the neutral peptides from YaYa and YaYc proteins. This shows that at least ten peptides (N1a–N10a and N1b–N10b) appear to be common to both proteins. YaYc protein also appeared to contain two peptides (N11b and N12b) that were not observed in YaYa protein. The results from the three pairs of analytical peptide 'maps' suggest that, although the number of soluble tryptic-digest peptides obtained from YaYa and YaYc proteins differed (32 and 38 respectively), all the peptides obtained from YaYa proteins appear to be recovered from YaYc protein.

Peptide fractionation

The peptides from the tryptic digests of YaYa and YaYc proteins were fractionated by elution from

Sephadex G-25 (Fig. 6). A 'map' of the peptides from these two proteins was constructed by performing electrophoresis at pH 6.5 on each of the fractions eluted from Sephadex G-25 (Fig. 7). On the basis of this 'map', fractions from YaYa protein eluted in the regions 70–80 ml, 81–100 ml, 101–120 ml, 121–138 ml and 139–160 ml were separately combined (Figs. 6 and 7). The fractions from YaYc protein eluted in the regions 70–80 ml, 81–98 ml, 99–120 ml, 121–136 ml and 137–160 ml were similarly combined (Fig. 6). The peptides in these pooled fractions were subsequently purified by a combination of electrophoresis at pH 6.5, descending chromatography and electrophoresis at pH 3.5 as described above.

The composition of these peptides was determined by electrophoresis at pH 2.1. No differences were noted in the composition or N-termini of any of the 32 peptides that appeared, from the peptide 'maps', to be common to both proteins. This suggests that YaYa and YaYc proteins possess substantial sequence identity.

Fig. 3. Two-dimensional 'map' of the acidic peptides from tryptic digests of YaYa and YaYc proteins

The tryptic digests of portions (1.5 mg of protein) of YaYa and YaYc proteins were prepared, the peptides were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and the acidic peptides were cut out of the paper. These peptides were separated in the second dimension by high-voltage electrophoresis at pH 3.5. Experimental details are given in the text. The peptides from YaYa protein are on the right-hand half of the 'map', and the peptides from YaYc protein are on the left-hand half of the 'map'.

Subdigest of tryptic 'core' from YaYa and YaYc proteins

The 'map' of the trypsin, V8 proteinase and chymotrypsin digests of the core material revealed that these enzymes released identical peptides from both proteins. Trypsin, V8 proteinase and chymotrypsin released nine, 12 and 11 peptides respectively.

Urea denaturation of YaYc protein and CM-Sephadex chromatography of renatured products

The subunits from YaYc protein were dissociated in 7M-urea and renatured by dialysis as described above. Material that was precipitated during dialysis was removed by centrifugation and analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. About 85% of this material comprised Ya monomer and 15% Yc monomer. About 60% of the original 1-chloro-2,4-dinitrobenzene-reduced glutathione-conjugating activity was recovered in the soluble fraction after the urea treatment.

The renatured products were analysed by CM-Sephadex chromatography (Fig. 8). Three peaks of enzyme activity were eluted at Na^+ concentrations of 40, 56 and 85 mM, which correspond to the elution positions of ligandin, transferase B and transferase AA from CM-Sephadex respectively (Hayes *et al.*, 1979, 1980). Analysis of these transferases by electrophoresis and Sephadex G-75 chromatography showed that these peaks contained respectively YaYa, YaYc and YcYc proteins (Fig.

Fig. 4. Two-dimensional 'map' of the basic peptides from tryptic digests of YaYa and YaYc proteins

The tryptic digests of portions (1.5 mg of protein) of YaYa and YaYc proteins were prepared as described in the text. The peptides were separated by high-voltage paper electrophoresis at pH 6.5 in the first dimension and at pH 3.5 in the second dimension. The peptides from YaYa protein are on the right-hand half of the 'map', and the peptides from YaYc protein are on the left-hand half of the 'map'.

Fig. 5. Two-dimensional 'map' of the neutral peptides from tryptic digests of YaYa and YaYc proteins

The tryptic digests of portions (1.5 mg of protein) of YaYa and YaYc proteins were prepared as described in the text. The single spot of neutral peptides obtained from each of YaYa and YaYc proteins after high-voltage paper electrophoresis at pH 6.5 (3.17) was separated in the first dimension by high-voltage paper electrophoresis at pH 3.5. These peptides were further resolved by descending chromatography with butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 16 h in a second dimension. The peptides from YaYa protein are on the right-hand half of the 'map', and the peptides from YaYc protein are on the left-hand half of the 'map'.

9). This is consistent with their identification as ligandin and transferases B and AA. The YaYc protein from the batch that was used for the urea denaturation (but was not subjected to urea denaturation) was eluted from CM-Sephadex as a single peak at an Na^+ concentration of 56 mM. This served as a control. Electrophoresis showed equal recoveries of Ya and Yc monomers from all the enzyme-containing fractions that comprised this peak, indicating that no spontaneous conversion of monomers occurred during either dialysis or chromatography (Fig. 9).

The Yc subunit was more resistant than the Ya subunit to urea treatment, since the Ya monomer comprised 85% of the material that was precipitated during dialysis. This unequal recovery of the two monomers (in a soluble form) after dialysis accounts for the proportions of YaYa/YaYc/YcYc eluted from CM-Sephadex being 1:2:2 rather than the expected 1:2:1.

Fig. 6. Fractionation of tryptic-digest peptides from YaYa and YaYc proteins

The tryptic digests of YaYa and YaYc proteins were prepared as described in the text. The digests were freeze-dried and redissolved in 2 ml of 50% (v/v) formic acid. This material was eluted (10 ml/h, 20°C) from three identical columns of Sephadex G-25 with 5% (v/v) formic acid. The three columns (270 cm × 1 cm) were used in series and 2 ml fractions were collected. The A_{280} of each fraction from the YaYa-protein digest (a) and from the YaYc-protein digest (b) was measured. The peptide content of each fraction was analysed by high-voltage paper electrophoresis at pH 6.5 (Fig. 7), and on the basis of the resulting 'map' the eluate from each protein digest was combined as indicated by the arrows. This resulted in the peptides from each digest being divided into five fractions to facilitate further purification.

Lithocholate binding to homodimers formed from YaYc protein

The homodimers were obtained from YaYc protein as described above. Lithocholate binding to these preparations was analysed by Sephadex G-75 chromatography under non-equilibrium conditions.

Fig. 7. Analysis of peptides obtained from tryptic digests of YaYa and YaYc proteins eluted from Sephadex G-25. Tryptic digests of YaYa and YaYc proteins were eluted from Sephadex G-25 (superfine grade) as described in the text. Fractions of volume 2 ml were collected, and 0.2 ml portions were removed from fractions 35–85 and desiccated. The dried material from the two proteins was redissolved in 25 μ l of 50% (v/v) pyridine and analysed in parallel by high-voltage paper electrophoresis at pH 6.5. The cathode is at the top and the anode is at the bottom. The resulting 'map' is 55 cm $\times$ 45 cm. The origin is indicated by the broken line (right to left). All the peptides to the right of the continuous line (in the centre from top to bottom) are from YaYa protein and the peptides to the left are from YaYc protein. Column fractions 41–80 from YaYa protein were applied sequentially (0.5 cm apart) from right to left, at the origin. Column fractions 41–80 from YaYc protein were similarly applied from right to left.

This demonstrated that only Ya-containing protein bound lithocholate. Neither the native YcYc protein (peak 5 in Fig. 1) nor the Yc homodimer obtained from YaYc protein bound this bile acid.

Discussion

Since the relationship between YaYa and YaYc proteins is not clear, we have investigated several mechanisms that have been reported to cause

interconversion of these two transferases *in vitro*. The peptide 'maps' from YaYa and YaYc proteins were compared. The possibility that the Ya subunits possessed by both proteins are identical was investigated. Bile acid binding to the individual Ya and Yc subunits was studied.

YaYa and YaYc proteins were resolved from whole cytosol by CM-Sephadex chromatography. Proteolysis was minimized in these experiments by (1) the use of Trasylol and (2) shortening the time

Fig. 8. Elution pattern of urea-treated YaYc protein from CM-Sephadex

The subunits of YaYc protein were dissociated by 7M-urea and reassociated as described in the text. The soluble products were applied to CM-Sephadex. Fractions (3.2 ml) were collected and the Na^+ concentration (■) and enzyme activity (▲) were measured. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on enzyme-containing fractions to determine the subunit compositions of the products. These are indicated.

before chromatography from 11 h to 2 h by omitting the dialysis step that we previously used (Hayes *et al.*, 1979). The elution profile obtained under these conditions was similar to that we have previously published, suggesting that proteolysis *in vitro* is not responsible for the recovery of the different transferases. This agrees with the findings obtained by other workers who used phenylmethanesulphonyl fluoride as a proteinase inhibitor (Habig *et al.*, 1974b; Bhargava *et al.*, 1978).

We have also analysed portions of YaYa and YaYc proteins by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis for evidence of inter-conversion *in vitro*. No change in the subunit composition of the proteins (which had been stored at -10°C for 9 months) was observed. Similarly, Bass *et al.* (1977) found no alteration in the subunit compositions of these proteins with storage (at 4°C for 6 months). Ketterer and his co-workers have also failed to find any evidence of spontaneous inter-conversion between YaYa and YaYc proteins (T. J. Carne, D. J. Meyer & B. Ketterer, personal communication). These results support the hypothesis that YaYa and YaYc proteins are stable separate proteins.

The suggestion made by Scully & Mantle (1981) that YaYa protein is formed as a purification artifact was based on apparent differences in the recovery of YaYa protein from CM-Sephadex and CM-cellulose. However, the elution profiles of the

transferases from these two ion-exchangers are not identical. A direct comparison between the elution of dialysed cytosol from one exchanger with the elution of undialysed cytosol from the other exchanger is not valid. Indeed, when dialysed cytosol is applied to CM-cellulose, YaYa protein is not retained but is eluted in the void volume (J. D. Hayes, R. C. Strange & I. W. Percy-Robb, unpublished work). When proposing that YaYa protein is formed as the result of proteolysis *in vitro*, Scully & Mantle (1981) only considered the subunit compositions of the transferases retained by CM-cellulose; they did not publish the subunit compositions of the void-volume enzyme peak. Further, the elution of YaYa protein in the first transferase-containing peak from CM-cellulose explains the unexpected finding by Hales & Neims (1976) that enzyme in this peak is precipitated by antisera raised against glutathione S-transferase B (YaYc protein).

The observed difference in molecular weight between YaYa and YaYc proteins is unlikely to be due to glycosylation, as no glucosamine or galactosamine was recovered from either protein. Further, no change in the subunit composition of YaYa and YaYc proteins was observed after incubation with neuraminidase, α -mannosidase and *N*-acetylglucosaminidase, which catalyse the removal of sialic acid, mannose, glucosamine and galactosamine.

The peptide 'maps' indicated that both proteins possess substantial identity. All the soluble peptides

Fig. 9. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of products from urea-treated YaYc protein

Native YaYc protein was eluted from CM-Sephadex as described in the text, and fractions 37–40, 42–45 and 47–50 were separately combined. Urea-treated YaYc protein was eluted from CM-Sephadex (Fig. 8), and fractions 33–36, 42–45 and 61–64 were combined. The samples were applied, for electrophoresis, from left to right: 1, YaYc protein; 2–4, fractions 37–40, 42–45 and 47–50 obtained from CM-Sephadex chromatography of native YaYc protein; 5–7, fractions 33–36, 42–45 and 61–64 obtained from CM-Sephadex chromatography of urea-treated YaYc protein. The samples (5–15 µg of protein) were run from the cathode (top) to the anode (bottom). The Ya and Yc polypeptides are indicated.

obtained from YaYa protein were also obtained from YaYc protein. Urea dissociation of YaYc protein, followed by renaturation, demonstrated that YaYa protein was formed as one of the products. The YaYa protein obtained from urea-treated YaYc protein possessed lithocholate-binding activity and was eluted in a manner similar to native YaYa protein from CM-Sephadex and Sephadex G-75. These experiments constitute strong evidence that the Ya subunits in both proteins are identical.

CM-Sephadex chromatography of the renatured products from urea-treated YaYc protein showed

that YaYc protein is a hybrid of ligandin (YaYa protein) and transferase AA (YcYc protein).

Hayes *et al.* (1980) showed that YaYa, YaYc and YcYc proteins all bind the primary bile acid, cholate, under equilibrium conditions. The data in the present paper demonstrate that under non-equilibrium conditions only YaYa and YaYc proteins bind the secondary bile acid, lithocholate; YcYc protein did not bind this bile acid under non-equilibrium conditions. The Ya subunit therefore contains a high-affinity binding site for lithocholate, whereas both Ya and Yc subunits possess a low-affinity binding site for cholate (which can only be demonstrated under equilibrium conditions). This finding that the Ya subunit possesses a high-affinity binding site explains the observation made by Strange *et al.* (1977) that lithocholate preferentially inhibited the enzyme activity of YaYa protein but not that of YaYc protein. Since cholate had no effect on the enzyme activity of YaYa or YaYc protein, the binding sites may be distinct.

In view of the apparent close homology between Ya and Yc subunits it is surprising that the Yc subunit fails to bind lithocholate. It is possible that the high-affinity domain exists in the Yc subunit but is inaccessible. The nature of this putative steric hindrance is not known, but it could be due to either conformational differences between the subunits or the presence of an 'activated' steroid that is covalently bound to a residue at the high-affinity binding site.

The lithocholate-binding activity of the hepatic Ya subunit varies from species to species. In human liver several different basic glutathione *S*-transferases are resolved by CM-Sephadex chromatography; these are all Ya homodimers. None of the human Ya homodimers binds lithocholate under non-equilibrium conditions (J. D. Hayes & D. B. Horn, unpublished work). In the trout (*Salmo gairdnerii*) a single basic hepatic glutathione *S*-transferase has been identified that comprises Ya subunits but does not possess lithocholate-binding activity (Nimmo *et al.*, 1981). Therefore, although in rat liver there appears to be only one form of the Ya monomer, which binds lithocholate, this cannot be assumed to be true in all species.

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