Purification and characterization of three forms of glutathione *S*-transferase A

A comparative study of the major YaYa-, YbYb- and YcYc-containing glutathione *S*-transferases

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Rat liver glutathione *S*-transferases have previously been defined by their elution behaviour from DEAE-cellulose and CM-cellulose as M, E, D, C, B, A and AA. These enzymes are dimeric proteins which comprise subunits of mol.wt. 22 000 (Ya), 23 500 (Yb) or 25 000 (Yc). Evidence is presented that YaYa protein, one of two previously described lithocholate-binding proteins which exhibit transferase activity, is an additional enzyme which is not included in the M, E, D, C, B, A and AA nomenclature. We therefore propose that this enzyme is designated transferase YaYa. Transferases YaYa, C, A and AA have molecular weights of 44 000, 47 000, 47 000 and 50 000 respectively and each comprises two subunits of identical size. These enzymes were purified to allow a study of their structural and functional relationships. In addition, transferase A was further resolved into three forms (A₁, A₂ and A₃) which possess identical activities and structures and appear to be the product of a single gene. Transferases YaYa, C, A and AA each had distinct enzymic properties and were inhibited by cholate. The recently proposed proteolytic model, which attributes the presence of multiple forms of glutathione *S*-transferase activity to partial proteolysis of transferase AA, was tested and shown to be highly improbable. Peptide maps showed significant differences between transferases YaYa, C, A and AA. Immunotitration studies demonstrated that antisera raised against transferases YaYa and C did not precipitate transferase AA.

The glutathione *S*-transferases are a group of detoxification enzymes in mammalian liver which represent 3–8% of the cytosolic protein. These enzymes have two well-described functions; they catalyse the conjugation of GSH with a large number of electrophilic compounds and bind a group of non-substrate hydrophobic ligands (Habig *et al.*, 1974a; Ketley *et al.*, 1975). The hepatic transferases in the rat are basic proteins that comprise two of three types of subunits (Ya, mol.wt. 22 000; Yb, mol.wt. 23 500; and Yc, mol.wt. 25 000) (Bass *et al.*, 1977). Jakoby and his colleagues (Habig *et al.*, 1974a, 1976) described the first purification scheme for the transferases in rat liver and proposed the currently used nomenclature; these

workers resolved seven transferases (M, E, D, C, B, A and AA) and purified the five quantitatively most important enzymes, namely E, C, B, A and AA. It is not clear whether this nomenclature is complete, because DCNB was originally used as the primary substrate to identify these enzymes, and it has subsequently been shown that not all transferases are active in conjugating this substrate with GSH.

A separate body of literature has arisen around ligandin, an organic-anion-binding protein in rat liver cytosol. Several purification schemes were established for ligandin (Litwack *et al.*, 1971) before it was demonstrated that ligandin possessed transferase activity (Kaplowitz *et al.*, 1973). Following the description of purified transferases it was reported that ligandin and transferase B were identical (Habig *et al.*, 1974b), and these terms were regarded as being synonymous. However, the relationship between ligandin and transferase B has recently been complicated, because it has been

Abbreviations used: SDS, sodium dodecyl sulphate; GSH, reduced glutathione; DCNB, 1,2-dichloro-4-nitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene.

recognized that there are two proteins that have been referred to as 'ligandin'. Different ligandin preparations comprise YaYa protein, YaYc protein or a mixture of these proteins (Carne *et al.*, 1979; Hayes *et al.*, 1979). This realization necessitates a re-evaluation of the ligandin literature. It is now apparent that transferase B (YaYc protein) is identical with one of the proteins referred to as 'ligandin', but it is not known which transferase corresponds to YaYa protein.

In addition to the confusion over the identity of YaYa protein, the relationship between the transferases is not clear. Controversy exists about the kinetic mechanism and the structure of transferase A, which was originally described by Jakoby and his colleagues as a microheterogeneous protein (Habig *et al.*, 1974a; Pabst *et al.*, 1974). However, these workers provided no evidence that the individual proteins in their preparation were functionally identical or were coded for by a single gene. In view of this lack of information it is possible that the different kinetic mechanisms for transferase A reported by Pabst *et al.* (1974) and Jakobson *et al.* (1977) are a result of these two groups studying either separate enzymes or different mixtures of distinct enzymes. Indeed, the fact that transferase A has been reported to comprise both Ya and Yc subunits (Listowsky *et al.*, 1976) and YbYb subunits (Hayes *et al.*, 1980; Scully & Mantle, 1980) supports this conclusion. A number of workers have reported that, during storage of transferase B (YaYc protein), the molecular weight of the Yc subunit is reduced from 25000 to 22000 (Listowsky *et al.*, 1976; Daniel *et al.*, 1977). Bhargava *et al.* (1978, 1980) and Ketterer *et al.* (1978) postulated that this phenomenon is due to the removal of 25–30 C-terminal amino acids from the Yc monomer and represents conversion of the Yc monomer into a Ya monomer. Scully & Mantle (1981) extended the Yc → Ya conversion hypothesis to include the Yb monomer(s) and proposed that all the transferases are generated by proteolysis of transferase AA (YcYc protein). This model has not, however, been tested.

We have investigated the structure and function of the major transferases in rat liver to resolve some of these anomalies. Experiments have been performed to establish which transferase corresponds to YaYa protein. Transferase A has been prepared, and the three proteins it comprises (A_1 , A_2 and A_3) have been separately purified. These enzymes have been characterized and compared with YaYa protein and transferases C and AA. Various approaches have been employed to enable the number of distinct polypeptides which these enzymes comprise to be determined and their functional and genetic relationships to be established. On the basis of these data we have proposed a modification to the

nomenclature which unambiguously describes this group of proteins in rat liver.

Materials and methods

Chemicals

The chemicals used were readily available commercially and were of analytical grade. Urea (Aristar) was from BDH Chemicals Ltd., Poole, Dorset, U.K. Cholic acid was from Maybridge Chemical Co., Tintagel, Cornwall, U.K., and was 99.5% pure. Aminoethyl-Sepharose 4B and α -chymotrypsin (type VII) were from Sigma Chemical Co., Poole, Dorset, U.K. Ampholines were from LKB Instruments Ltd., Selsdon, South Croydon, Surrey, U.K.

Enzyme assays

Glutathione S-transferase activity was measured at 37°C by using a centrifugal analyser (Rotochem Ila parallel fast analyser, American Instrument Co., Silver Spring, MD, U.S.A.). The standard assay for measuring the conjugation of both CDNB (1 mmol/litre) with GSH (2 mmol/litre) and DCNB (1 mmol/litre) with GSH (5 mmol/litre) was carried out in 100 mM-sodium phosphate buffer at pH 6.5 and 7.5 respectively. The samples were preincubated with GSH and the reactions (a maximum of 35 were analysed simultaneously) were initiated by centrifugation. Reactions were monitored by measuring the A_{340} at 10 s after mixing and subsequently on nine occasions at 5 s intervals. Reaction rates were determined by using the manufacturer's kinetic rate program to fit regression curves to the nine $\Delta A_{340}/5$ s values that were obtained from each cuvette.

SDS/polyacrylamide-gel electrophoresis

This was carried out by the method of Laemmli (1970) by using the LKB 2001 vertical electrophoresis unit. For analytical purposes and peptide-mapping experiments the resolving gel (0.15 cm × 15 cm × 15 cm) comprised 12.5 and 16.5% (w/v) polyacrylamide (containing 0.33 or 0.44% cross-linker) respectively.

Identification of YaYa protein

To enable the transferase that corresponds to YaYa protein to be identified, the transferases were purified by the definitive scheme of Habig *et al.* (1976) from single male Wistar or Sprague-Dawley rat livers (10–12 g). This involved DEAE-cellulose chromatography (which removed transferase M), fractionation with $(\text{NH}_4)_2\text{SO}_4$ and CM-cellulose chromatography. The subunit composition of fractions from CM-cellulose were analysed by SDS/polyacrylamide-gel electrophoresis and the lithocholic acid-binding activity of Ya-containing frac-

tions was examined by CM-Sephadex chromatography after the addition of [^{14}C]lithocholic acid (0.5 μCi , 8 nmol) (Hayes, 1980). The peak of transferase activity, from CM-cellulose, which, after rechromatography, eluted from CM-Sephadex at a Na^+ concentration of 40 mmol/litre (identical with the position at which purified YaYa protein is eluted), was analysed by SDS/polyacrylamide-gel electrophoresis to confirm its identification.

Purification of glutathione S-transferases

To allow the transferases to be characterized, large-scale purification of these enzymes was carried out by using established methods. YaYa protein was purified as previously described (Hayes, 1980). Transferases C and AA were purified, from 200 g of frozen rat liver, by the method of Habig *et al.* (1976). Briefly, this involved DEAE-cellulose chromatography, precipitation with $(\text{NH}_4)_2\text{SO}_4$, CM-cellulose chromatography and two hydroxyapatite steps. Transferase A was purified by a modification of the method of Habig *et al.* (1974a). The DEAE-cellulose, $(\text{NH}_4)_2\text{SO}_4$ and CM-cellulose steps were performed as described above, but a shallower gradient was employed for the hydroxyapatite chromatography than was used by Habig *et al.* (1974a). The first hydroxyapatite column was developed with a sodium phosphate gradient (10–250 mmol/litre) at pH 6.7 and resulted in the elution of three transferases with GSH–CDNB-conjugating activity. These were designated 'A₁', 'A₂' and 'A₃', by their order of elution, and each was resubjected to hydroxyapatite chromatography under identical conditions.

After the final hydroxyapatite step, transferases C, AA, A₁, A₂ and A₃ were further purified by cholic acid–aminoethyl-Sepharose 4B chromatography (Pattinson *et al.*, 1980). The purified enzymes were each dialysed (4°C, 20 h) against two changes, each of 2 litres, of 10 mM-sodium phosphate buffer, pH 6.7, containing GSH (1 mmol/litre) and EDTA (0.1 mmol/litre) before storage at –20°C.

Inhibition of transferase activity by urea

The effect of urea on the GSH–CDNB-conjugating activity of each transferase was determined by preincubating portions (1–3 μg of protein) with various concentrations of urea (0–4.5 mol/litre) for 40 min (20°C) before the reactions were started.

Inhibition of transferase activity by cholate

The effect of cholate on the GSH–CDNB-conjugating activity of each transferase was determined. The activity of YaYa protein and transferases C, A₁, A₂ and A₃ was measured with various concentrations of CDNB (0.03, 0.1, 0.3 and 1.0 mmol/litre) in the presence of cholate (0, 0.08, 0.16, 0.24, 0.41, 0.57 or 0.82 mmol/litre). The

activity of transferase AA was measured by using CDNB (0.1, 0.3 and 1.0 mmol/litre) in the presence of cholate (0, 0.24, 0.41, 0.82, 1.22 and 1.62 mmol/litre). Each experimental point was measured in quadruplicate and the mean value was used for further calculations. Estimates of K_m and $V_{\max}$ were calculated by using a least-squares non-linear fitting procedure of the mean velocity against CDNB concentration (Wilkinson, 1961). The inhibition constants of transferases A₁, A₂, A₃ and C were determined by using plots of $1/V_{\max}$ against cholate concentration and those of YaYa protein and transferase AA by using Dixon (1953) plots. The calculations and graphical output were programmed on a Hewlett–Packard 9821 desk calculator with printer/plotter by Dr. A. F. Smith of this Department.

Two-dimensional isoelectric focusing–SDS/polyacrylamide-gel electrophoresis

This was performed as described by O'Farrell (1975). Portions (about 0.5 mg) of the purified transferases were prepared for electrophoresis by precipitation with ice-cold 10% (w/v) trichloroacetic acid. The pellet obtained after centrifugation (60 min, 2°C, 5000 g) was redissolved in about 0.15 ml of sample buffer [9.5 M-urea/2% (v/v) Nonidet P40/5% (v/v) 2-mercaptoethanol/0.4% Ampholines pH 3.5–10/0.8% Ampholines pH 7–9/0.8% Ampholines pH 9–11]. The first-dimension gels comprised 9.5 M-urea/2% (v/v) Nonidet P40/0.4% Ampholines pH 3.5–10/0.8% Ampholines pH 7–9/0.8% Ampholines pH 9–11/5% (w/v) acrylamide (containing 5% cross-linker) and were cast in 0.3 cm $\times$ 12 cm glass tubes. The anode reservoir contained 1.25 litres of 0.01 M-phosphoric acid and the cathode reservoir, which was changed three times during each experiment, contained 0.4 litres of 0.02 M-NaOH. The gels were prefocused for 75 min (at 0.1 W/gel) before the samples were added. Twelve gels were run in parallel; protein samples were applied to nine gels and three served as controls (to enable the pH gradient to be determined). About 15 μg of each pure enzyme was applied to the gel, at the cathodal end, and 15 μl of overlay buffer (8 M-urea/0.2% Ampholines pH 3.5–10/0.4% Ampholines pH 7–9/0.4% Ampholines pH 9–11) was layered on top. Electrophoresis was carried out for 15 h at 400 V, followed by a final 1 h at 800 V. After electrophoresis the rod gels were extruded and the anodal end marked with Indian ink. The gels for the second dimension were equilibrated with SDS and 2-mercaptoethanol before being bonded to the top of the second-dimension gel using agarose. SDS/polyacrylamide-gel electrophoresis was used for the second dimension and is described above. The staining and destaining procedures have been described previously (O'Farrell, 1975).

Peptide mapping

This was carried out by the method of Cleveland *et al.* (1977). Portions of the purified transferases (0.13 mg in 0.25 ml of 25 mM-sodium phosphate buffer, pH 7.5) were prepared for digestion by heating (5 min, 95°C) after the addition of SDS and EDTA, to give final concentrations of 0.1% (w/v) and 0.1 mM respectively. These solutions were cooled on ice before the addition of 20 µg, 2 µg, 0.2 µg or 0.02 µg of α -chymotrypsin. Digestion was carried out at 37°C for 45 min and was stopped by heating at 95°C for 5 min after the addition of glycerol, SDS, 2-mercaptoethanol and Bromophenol Blue to give final concentrations of 10% (v/v), 1% (w/v), 2% (v/v) and 0.002% (w/v) respectively. The peptides were resolved by SDS/polyacrylamide-gel electrophoresis using a 16.5% resolving gel. The structural relationship between the Ya, Yb and Yc dimers was assessed by applying a statistical method of evaluating the significance of peptides of similar and distinct molecular weights from different proteins (Calvert & Gratzner, 1978).

Immunotitration

Antiserum against YaYa protein was raised in female New Zealand White rabbits by using standard immunological techniques. Antiserum against transferase C was a gift from Dr. W. B. Jakoby, Section on Enzymes and Cellular Biochemistry, National Institutes of Health, Bethesda, MD, U.S.A. Portions (4 µg) of the purified transferases were incubated in 100 mM-sodium phosphate buffer, pH 6.5, containing various amounts (5–160 µl) of antiserum. The final volume of the incubation mixtures was 0.6 ml. The reactions were allowed to proceed at 4°C for 40 h before centrifugation (40 min, 10000 g, 4°C). The remaining transferase activity in the supernatant was measured with CDNB as described above. The results are expressed as a percentage of the activity recovered after incubation with 160 µl of non-immune rabbit serum (i.e. control value).

Results

Enzyme assays

The GSH-DCNB- and GSH-CDNB-conjugating activities were measured by using a centrifugal analyser. To assess the precision of the enzyme assays, portions of partially purified transferase C were routinely re-analysed. The performance of the GSH-CDNB-conjugation assay was monitored by using controls possessing $\Delta A_{340}/\text{min}$ per ml values of approx. 30 and 100. Repeat analysis of these samples, during the present study, gave values of 31.3 ± 2.2 (mean $\pm$ s.d.) and 99.4 ± 4.2 (mean $\pm$ s.d.). The GSH-DCNB-conjugation assay

was monitored by using a control possessing a $\Delta A_{340}/\text{min}$ per ml value of approx. 40. Analysis of this material gave values of 40.3 ± 3.7 (mean $\pm$ s.d.).

Identification of YaYa protein

The initial experiments to determine which transferase corresponds to YaYa protein were carried out by using the single-rat-liver purification scheme of Habig *et al.* (1976). This method was chosen because it is the basis of the currently used nomenclature. Transferase M was removed by the initial purification step on DEAE-cellulose. Fig. 1 shows the elution of the transferases from CM-cellulose. The transferases were resolved into six peaks of GSH-CDNB-conjugating activity. These were numbered by their elution order (I–VI). Peak I eluted in the void volume of the column and peaks II–VI were eluted by a salt gradient at Na⁺ concentrations of 34, 44, 55, 63 and 71 mmol/litre respectively. Peaks I–VI were analysed by SDS/polyacrylamide-gel electrophoresis, which showed that only peaks II and IV contained significant concentrations of the Ya monomer. These two peaks were separately assayed for lithocholic acid-binding activity by using CM-Sephadex chromatography. Both peaks II and IV bound lithocholic acid, but were eluted differently from CM-Sephadex at Na⁺ concentrations of 40 and 57 mmol/litre respectively. The elution positions from CM-Sephadex suggested that peak II contained YaYa protein and that peak IV contained transferase B (Hayes *et al.*, 1979). This was confirmed by SDS/polyacrylamide-gel electrophoresis. The subunit compositions, the elution positions from CM-cellulose, the GSH-DCNB-conjugating activities and the lithocholic acid-binding activities suggest the peaks contain the following transferases: I, transferases D and E; II, YaYa protein; III, transferase C; IV, transferase B; V, transferase A; and VI, transferase AA.

The elution pattern from CM-cellulose was highly reproducible and did not change when the full-scale purification method was used. The identification of peaks III, IV, V and VI as transferases C, B, A and AA respectively was confirmed by their behaviour on hydroxyapatite. During the full-scale purification peaks III, IV, V and VI from CM-cellulose were separately subjected to hydroxyapatite chromatography and were eluted at Na⁺ concentrations of 275, 240, 300–340 and 260 mmol/litre respectively. These elution positions from hydroxyapatite are consistent with our identification of the transferase peaks (Habig *et al.*, 1976; Guthenberg & Mannerik, 1979).

Purity of enzyme preparations and subunit compositions

The transferases were purified as described above.

Transferase A was resolved into three peaks of GSH-DCNB-conjugating activity by hydroxyapatite chromatography; these eluted at Na^+ con-

centrations of 300, 325 and 340 mmol/litre and were designated 'transferase A_1 ', ' A_2 ' and ' A_3 ' respectively (Fig. 2). These transferases were separately

Fig. 1. *Elution pattern of glutathione S-transferases from CM-cellulose*

The soluble extract (40 ml, 2 g of protein) from a frozen rat liver was applied to a DEAE-cellulose column (2.2 cm $\times$ 21.0 cm) equilibrated and eluted with 10 mM-Tris/HCl buffer, pH 8.1. The material which eluted between 35 and 95 ml was combined, concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and dialysed against 4 litres of 10 mM-sodium phosphate buffer, pH 6.7 (16 h, 4°C). The dialysed solution (12 ml, 230 mg of protein) was applied to a CM-cellulose column (2.2 cm $\times$ 15.0 cm). The flow rate was 20 ml/h and the fraction volume was 3.3 ml. The Na^+ concentrations ($\square$) were determined by flame photometry, and transferase activity was measured with CDNB (Δ) or DCNB ($\circ$) as described in the text.

Fig. 2. *Elution pattern of partially purified transferase A from hydroxyapatite*

The transferases were purified from 200 g of frozen rat liver as described in the text. Transferase A-containing fractions which eluted from CM-cellulose (peak V) were combined and dialysed against 4 litres of 10 mM-sodium phosphate buffer, pH 6.7 (16 h, 4°C). The dialysed material (30 ml, about 250 mg of protein) was applied to an hydroxyapatite column (2.2 cm $\times$ 14.0 cm). Fractions (4.9 ml) were collected. Glutathione S-transferase activity, with CDNB (Δ) and DCNB ($\circ$), and Na^+ concentrations ($\square$) were measured. The fractions under the bars were combined separately, dialysed against 10 mM-sodium phosphate buffer, pH 6.7, and individually applied to another column (2.2 cm $\times$ 14.0 cm) of hydroxyapatite.

combined and, after rechromatography on hydroxyapatite, each was eluted as a single peak of transferase activity from hydroxyapatite. SDS/polyacrylamide-gel electrophoresis showed that each of the preparations of YaYa protein and transferases C, A₁, A₂, A₃ and AA was at least 97% pure. Transferases A₁, A₂ and A₃ each comprised two subunits of mol.wt. 23 500 (Yb), which co-migrated with the subunits of transferase C (Fig. 3).

Apparent K_m values

The K_m values of transferases A₁, A₂ and A₃ for both GSH and CDNB were identical. However,

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the purified glutathione *S*-transferases

The transferases were purified and analysed by SDS/polyacrylamide-gel electrophoresis as described in the text. Portions of each protein (10–15 μ g) were applied to the gel as follows: (1) YaYa protein; (2) transferase C; (3) transferase B; (4) transferase A₁; (5) transferase A₂; (6) transferase A₃ and (7) transferase AA. The samples were run from the cathode (top) to the anode (bottom).

each of the remaining enzymes (YaYa protein and transferases C and AA) exhibited characteristic Michaelis constants for these substrates (Table 1).

Inhibition of transferase activity

Fig. 4(a) shows that the activity of transferases A₁, A₂ and A₃ cannot be distinguished by the use of urea. A linear relationship exists between the decrease in activity of transferases C, A₁, A₂ and A₃ and the concentration of urea used during the preincubation. In contrast, Fig. 4(b) shows that a plot of the activity of YaYa protein and transferase AA against urea concentration was biphasic.

Cholate inhibited the activity of all the enzymes studied. The effect of cholate on the activity of transferases A₁, A₂ and A₃ was identical. Both the apparent K_m and V_{max} decreased as the inhibitor concentration increased. These data fit the criteria of mixed inhibition. Plots of $1/V_{max}$ against cholate concentration enabled the K_i values to be calculated (Table 2). Although the fit of the straight line through these points appeared good, the $1/V_{max}$ values were slightly lower than expected at high cholate concentrations. This resulted in a small curvature of the data points towards the x -axis, indicating that the reaction can never be shut off completely. This may reflect a slow, but finite, breakdown in the enzyme–inhibitor–substrate complex or the formation of cholate micelles. Transferase C activity was also inhibited by a mixed mechanism which was indistinguishable from the type of inhibition affecting transferases A₁, A₂ and A₃. In contrast, the V_{max} of YaYa protein remained constant at different inhibitor concentrations, suggesting that cholate inhibits the transferase activity of YaYa protein by a competitive mechanism. Transferase AA was not inhibited by cholate as readily as the other enzymes; at a cholate concentration of 0.82 mmol/litre (and GSH and CDNB concentrations of 2 and 1 mmol/litre respectively), transferase AA activity was decreased to 93% of that obtained in the absence of cholate, whereas activities of transferases C, A₁, A₂ and A₃ and YaYa

Table 1. Elution characteristics, subunit molecular weights and Michaelis constants of the major glutathione *S*-transferases

K_m values were determined at pH 6.5 and results are means $\pm$ S.E.M.

Transferase	Elution position from CM-cellulose (peak no.)	$10^{-3} \times$ Subunit mol.wt.	K_m (mM)	
			GSH	CDNB
YaYa protein	II	22	0.771 ± 0.075	0.949 ± 0.125
C	III	23.5	0.321 ± 0.015	0.173 ± 0.010
A ₁	V	23.5	0.217 ± 0.010	0.105 ± 0.001
A ₂	V	23.5	0.209 ± 0.017	0.096 ± 0.012
A ₃	V	23.5	0.245 ± 0.015	0.101 ± 0.027
AA	VI	25	0.584 ± 0.051	0.969 ± 0.006

Fig. 4. Inhibition of transferase activity by urea. Portions of each transferase were incubated with various amounts of urea at pH 6.5. After 40 min the activity remaining was measured as described in the text. Each point represents the mean of four determinations and is expressed as a percentage of the activity obtained in the absence of urea. The

protein were decreased to 34, 24, 25, 27 and 62% respectively. Likewise, at a cholate concentration of 1.62 mmol/litre, transferase AA activity was decreased to 83% and activities of transferases C, A₁, A₂, A₃ and YaYa protein were decreased to 24, 15, 16, 18 and 44% respectively. The effect of cholate on transferase AA is peculiar; although the enzyme activity was inhibited, there was an apparent increase in both K_m (for CDNB) and V_{max} with increasing cholate concentrations. Cholate may therefore affect transferase AA by several mechanisms; it may exert a direct or indirect inhibitory effect at the active centre while simultaneously affecting, through its detergent activity, the solubility of the enzyme.

Isoelectric focusing

Transferases A₁, A₂, A₃, AA and C and YaYa protein were reduced with 2-mercaptoethanol and subjected to gel isoelectric focusing in the presence of 9.5 M-urea. Under these denaturing conditions each transferase focused as a single band. Transferases A₁, A₂ and A₃ were indistinguishable using this technique and possessed isoelectric points (pI) of 8.9. YaYa protein and transferase AA both had a pI value of 9.0. Transferase C had a pI value of 8.3. In addition, a faint band (which accounted for less than 5% of the total protein) was occasionally detected after electrofocusing of transferase C, which had a pI of 8.6. The significance of this minor component is not clear, but since it had a mol.wt. of 23 500 and was not always observed, it may be due to a modification of this protein during electrophoresis. Fig. 5(a) shows a two-dimensional electrophoretic map of transferases C and A₂. Fig. 5(b) shows a map of the polypeptides that YaYa protein and transferases C, A₂ and AA comprise.

Peptide mapping

Peptide maps of transferases A₁, A₂ and A₃ were constructed to determine whether these enzymes are genetically distinct or are variants of a single protein. Digestion of transferases A₁, A₂ and A₃ with chymotrypsin yielded identical polypeptide patterns. A total of 14 polypeptides of mol.wts. 21 000, 20 900, 19 600, 19 400, 18 200, 17 400, 16 300, 16 100, 15 600, 15 100, 14 100, 13 000, 10 900 and 10 000 were obtained from each enzyme (Fig. 6). Peptide maps of transferases A₁, A₂ and A₃ were compared with that of transferase AA to determine whether these enzymes could be generated by proteolysis of YcYc protein. Digestion of transferase AA gave 12 polypeptides; the molecular

effect of urea on the GSH-CDNB-conjugating activity of transferase is shown: (a) Δ , A₁; $\circ$, A₂; $\square$, A₃, (b) $\bullet$, C; $\blacksquare$, AA; $\blacktriangle$, YaYa protein.

Table 2. *Cholate inhibition of glutathione S-transferases*

Transferase	$10^{-3} \times$ Subunit mol.wt.	$10^3 \times K_i$ (M)	Type of inhibition
YaYa protein	22	0.55	Competitive
C	23.5	0.40	Mixed
A ₁	23.5	0.26	Mixed
A ₂	23.5	0.27	Mixed
A ₃	23.5	0.30	Mixed
AA	25	2.50	*

* Both the K_m for CDNB and V_{max} increased with increasing cholate concentrations.

Fig. 5. *Two-dimensional electrophoresis of the transferases*

Portions (15 μ g) of (a) transferases C and A₂ and (b) YaYa protein and transferases C, A₂ and AA were subjected to two-dimensional electrophoresis as described in the text. The pH gradient and the Ya, Yb and Yc monomers are indicated.

weight of these was 23 500, 22 000, 18 800, 17 900, 16 600, 16 000, 15 400, 14 600, 13 600, 13 100, 12 400 and 10 400. The 16 000-, 13 100- and 12 400-mol.wt. fragments were major digestion

Fig. 6. *Partial proteolysis of transferases A₁, A₂ and A₃*. Portions (130 μ g) of transferases A₁, A₂ and A₃ were digested (37°C, 45 min) with 2 μ g of chymotrypsin. The samples were applied as follows: (1) 5 μ g of lysozyme; (2) 50 μ g of transferase A₁ digest; (3) 50 μ g of transferase A₂ digest; (4) 50 μ g of transferase A₃ digest; and (5) 5 μ g of lysozyme. The Yb band is indicated.

intermediates, which were represented over a wide range of substrate/chymotrypsin ratios. Although the transferase AA maps contained a 23 500-mol.wt. polypeptide, it is highly unlikely that transferase A₁, A₂ or A₃ has arisen as the result of proteolysis, since all the remaining fragments are distinct from those of transferases A₁, A₂ and A₃.

To allow a comprehensive comparison between the transferases, and to determine whether either YaYa protein or transferase C is formed from transferase AA, peptide maps of transferase C and YaYa protein were constructed and compared with the peptide map of transferase AA. There was a marked difference between the map of transferase AA and YaYa protein (Fig. 7). Seven polypeptides, with mol.wts. 21 700, 21 600, 14 300, 13 500, 12 600, 11 600 and 11 200, were observed from YaYa protein. None of these were obtained from transferase AA, and although transferase AA yielded a 22 000-mol.wt. fragment, a statistical method of comparing digest patterns (Calvert & Gratzer, 1978) indicated that the Ya and Yc monomers are coded

Fig. 7. Partial proteolytic digestion of YaYa protein and transferase AA

Portions ($130\mu\text{g}$) of YaYa protein and transferase AA were digested with various amounts of chymotrypsin. After 45 min digestion the mixtures were analysed by SDS/polyacrylamide-gel electrophoresis. The samples were applied as follows: (1) $7\mu\text{g}$ of chymotrypsin; (2–5) $50\mu\text{g}$ of YaYa protein digests containing $0.007\mu\text{g}$, $0.07\mu\text{g}$, $0.7\mu\text{g}$ and $7\mu\text{g}$ of chymotrypsin respectively; (6–9) $50\mu\text{g}$ of transferase AA digests containing $7\mu\text{g}$, $0.7\mu\text{g}$, $0.07\mu\text{g}$ and $0.007\mu\text{g}$ of chymotrypsin respectively; and (10) $7\mu\text{g}$ of chymotrypsin.

Fig. 8. Partial proteolytic digestion of transferases C and AA

Portions ($130\mu\text{g}$) of transferases C and AA were digested with various amounts of chymotrypsin. After 45 min digestion the mixtures were analysed by SDS/polyacrylamide-gel electrophoresis. The samples applied were: (1) $7\mu\text{g}$ of chymotrypsin; (2–5) $50\mu\text{g}$ of transferase C digests containing $0.007\mu\text{g}$, $0.07\mu\text{g}$, $0.7\mu\text{g}$ and $7\mu\text{g}$ of chymotrypsin respectively; (6–9) $50\mu\text{g}$ of transferase AA digests containing $7\mu\text{g}$, $0.7\mu\text{g}$, $0.07\mu\text{g}$ and $0.007\mu\text{g}$ of chymotrypsin respectively; and (10) $7\mu\text{g}$ of chymotrypsin.

separately. The map of transferase C was also clearly distinct from that of transferase AA (Fig. 8). Fragments with mol.wts. 21000, 20900, 19600, 19400, 18200, 17400, 16300, 16100, 15600, 15100, 14100, 13000, 10900 and 10000 were observed from transferase C. The digests of YaYa protein and transferases C, A₂ and AA were also performed in parallel to permit a direct comparison between all the peptide maps (Fig. 9).

Although there is considerable structural similarity between transferases A₁, A₂, A₃ and C, these enzymes are clearly distinct from transferase AA and YaYa protein. Minor differences exist between the peptide maps of transferase C and those of transferases A₁, A₂ and A₃. The 14100- and 15100-mol.wt. fragments from transferase C were intense easily recognizable bands, but were only faintly represented in the transferase A₁, A₂ and A₃ digests. Conversely, the 16100-mol.wt. fragment is quantitatively a more important degradative intermediate in transferases A₁, A₂ and A₃ than in transferase C. Although the significance of these differences is not known, they are entirely reproducible and were not due to the time of sampling the digests.

Discussion

Although the original description of the glutathione *S*-transferases by Jakoby and his colleagues (Habig *et al.*, 1974a, 1976) was considered comprehensive, recent work has shown that this may not be the case: the identity of YaYa protein is unknown, and transferase A may contain several enzymes. In addition, the relationship between the transferases is not clear, since a number of workers have postulated that various enzymes are interconvertible. These problems merit investigation as they represent major obstacles to an understanding of the structural/functional relationship between members of this group of enzymes.

The method of Habig *et al.* (1976) was used to identify YaYa protein because it represents the definitive purification scheme for the transferases. By using this protocol, YaYa protein was separated from transferase M by DEAE-cellulose chromatography, and it was eluted, with transferases E, D, C, B, A and AA, in the void volume of the DEAE-cellulose column. This was followed by CM-cellulose chromatography, which resulted in YaYa protein being eluted after transferases D and E (peak

Fig. 9. Partial proteolysis of glutathione *S*-transferases. Portions (130 μ g) of YaYa protein and transferases C, A₂ and AA were digested (37°C, 45 min) with 2 μ g of chymotrypsin. The samples subjected to SDS/polyacrylamide-gel electrophoresis were: (1) 5 μ g of lysozyme; (2) 50 μ g of YaYa protein digest; (3) 50 μ g of transferase C digest; (4) 50 μ g of transferase A₂ digest; (5) 50 μ g of transferase AA digest; and (6) 5 μ g of lysozyme.

I), but before transferases C, B, A and AA (peaks III–VI) (Fig. 1). Although Habig *et al.* (1974a) reported that peak I contained transferases D and E, it is difficult to demonstrate that YaYa protein (peak II) is an additional, previously unidentified, transferase, since neither transferase D nor E has been well-characterized. However, a number of facts suggest that YaYa protein is distinct from transferases D and E. Firstly, in all our CM-cellulose elution profiles the GSH–CDNB and GSH–DCNB-conjugating activities in peak I were not superimposable, suggesting the presence of two transferases in the void-volume peak (i.e. D and E). Secondly, the pI value of native YaYa protein is distinct from that of transferase E (Fjellstedt *et al.*, 1973; Hayes, 1980). Thirdly, Jakob *et al.* (1976) showed that transferase D can be distinguished from the other transferases by its ability to catalyse the conjugation of GSH with *p*-nitrophenethyl bromide; when cytosol was eluted from CM-Sephadex the major peak of GSH–*p*-nitrophenethyl bromide-con-

jugating activity was not eluted with YaYa protein but with the first two peaks that appeared before the salt gradient (J. D. Hayes, unpublished work). Fourthly, Jakob & Habig (1980) have shown that transferases D and E are quantitatively unimportant (they represent 1 and 4% respectively of the rat liver transferases), whereas YaYa protein is present in relatively high concentrations in normal rats (it accounts for at least 18% of hepatic transferases) and after phenobarbitone treatment it represents the major transferase. Fifthly, preliminary data suggests that transferases D and E are Yb homodimers (Ketterer *et al.*, 1982). These data are consistent with the hypothesis that YaYa protein is an additional transferase.

Recently, Scully & Mantle (1981) resolved a transferase from the cytosol of various tissues which comprised Ya subunits and was called 'transferase X'. As these workers did not use the purification methods described by Habig *et al.* (1974a, 1976), they were unable to relate this enzyme to the existing transferase nomenclature. Transferase X eluted from CM-cellulose at a position equivalent to YaYa protein and we conclude that these enzymes are identical. In contrast with Scully & Mantle (1981), we found YaYa protein in every liver specimen examined (at least 20), whereas those workers only occasionally observed transferase X in their preparations. This apparent difference in the recovery of YaYa protein is probably due to inconsistencies in its elution position from various ion-exchangers rather than partial proteolysis *in vitro*. The elution position of YaYa protein from CM-cellulose is variable and is dependent on the protein concentration of the sample applied (Hayes *et al.*, 1981).

Since ligandin preparations have been shown to comprise YaYa protein and/or YaYc protein, some workers also refer to glutathione *S*-transferase B as a mixture of these two proteins. This is incorrect, as transferase B comprises only YaYc protein (Hayes *et al.*, 1979). The term ligandin is ambiguous and should not be used unless the subunit composition of the preparation is defined. To avoid such confusion we propose that YaYa protein be designated glutathione *S*-transferase YaYa.

Our data are in agreement with those of Kalinyak & Taylor (1982), who, using wheat-germ and rabbit reticulocyte translation systems, have also proposed that transferase YaYa and transferase B are separate enzymes.

The structure and function of the different forms of transferase A were investigated because of the controversy, described in the introduction, surrounding this enzyme. Pabst *et al.* (1974) demonstrated that transferase A preparations contained three proteins, but these have not been studied separately and their genetic relationship is not

known. In the present study these three enzymes were resolved by hydroxyapatite chromatography and designated A₁, A₂ and A₃ by their order of elution. We were unable to distinguish between the activities of transferases A₁, A₂ and A₃; each enzyme possessed identical K_m values for GSH and CDNB and was similarly inhibited by urea or cholate. Structural investigation, using SDS/polyacrylamide-gel electrophoresis, isoelectric focusing and peptide mapping, also showed that these enzymes are indistinguishable. Indeed, the only difference detected between transferases A₁, A₂ and A₃ was their distinct elution positions from hydroxyapatite. The three forms of transferase A probably arise as the result of autoxidation, since when mercaptoethanol (2 mmol/litre) is included in the buffers, transferases A₁ and A₂ elute from hydroxyapatite at the position occupied by transferase A₃. Conversely, repetitive chromatography or extensive dialysis of transferases A₂ and A₃ in the absence of mercaptoethanol results in these enzymes eluting from hydroxyapatite at the position occupied by transferase A₁. It is concluded that these enzymes represent different forms of a single gene product. However, it is not clear whether this putative autoxidation occurs *in vivo* as well as *in vitro*. In contrast with Listowsky *et al.* (1976), we found no evidence of Ya and/or Yc monomers in our transferase A preparations. These were all Yb homodimers. The differences in the kinetic mechanism proposed by Pabst *et al.* (1974) and Jakobson *et al.* (1977) for transferase A are not the result of these workers studying distinct enzymes but are probably due to different assumptions being made about the number of GSH-binding sites per polypeptide.

The possibility that the transferases are formed from transferase AA (Scully & Mantle, 1981; Grover, 1982) was investigated by comparing the activities and the peptide maps of each enzyme. The Michaelis constants for GSH and CDNB and the inhibitory effect of cholate showed that transferases YaYa, C, A and AA have distinct activities. Presumably the active centre of transferase AA differs from those of the other enzymes studied. The peptide maps were constructed by using partial proteolysis. This was considered to be the preferred technique because total digestion of the transferases results in the formation of an insoluble 'core', which cannot be analysed by conventional peptide-mapping methods (Ketterer *et al.*, 1976; Hayes *et al.*, 1981). Analysis of the limited digests by SDS/polyacrylamide-gel electrophoresis enabled a number of peptide maps to be constructed in parallel, facilitating a direct comparison between transferases YaYa, C and A and transferase AA. These maps show that it is highly improbable that transferase AA is a parental enzyme which can be converted, after partial proteolysis, into transferases YaYa, B, C and A. According to the proteolytic model, transferases YaYa and C represent the final functional degradative products at the end of two alternative pathways. This model would predict that all the antigenic determinants which characterize transferase YaYa and transferase C should also be present in transferase AA. Indeed, all the transferases might be expected to cross-react with antisera raised against an individual enzyme. This was not found. Immunotitration of these enzymes revealed that transferase AA was not precipitated by antisera raised against transferases YaYa or C (Table 3).

Table 3. *Immunotitration of transferases YaYa, C, A and AA*

The immunotitrations were carried out as described in the text. The results are expressed as a percentage of the control values.

Transferase	Volume (μ l) of anti-(transferase YaYa) serum added														
	...	0	5	10	15	20	25	30	35	40	45	50	55	60	
YaYa		100	81	72	64	56	49	39	31	22	14	10	3	1	
C		100	99	100	101	101	102	101	99	98	98	97	95	95	
A*		100	104	104	104	100	100	100	96	95	96	97	95	110	
AA		100	103	101	100	98	97	95	91	91	89	88	89	87	

Transferase	Volume (μ l) of anti-(transferase C) serum added												
	...	0	5	10	15	20	30	40	60	80	120	160	
YaYa		100	94	103	98	102	99	102	102	100	98	88	
C		100	80	61	48	34	21	16	13	11	8	7	
A*		100	89	72	58	41	26	20	14	10	5	4	
AA		100	96	100	100	99	99	98	100	97	95	92	

* The transferase A preparation was obtained in the presence of mercaptoethanol (2 mmol/l) and therefore contains transferases A₁, A₂ and A₃.

The present study raises the question concerning the relationship between transferases A and C, since there are obvious similarities between these proteins. The peptide maps suggest that there is substantial sequence homology between the different forms of transferase A and transferase C. Although a product-precursor relationship may exist between these two groups of enzymes, we think that they are genetically distinct for several reasons. Firstly, although the differences between the peptide maps of transferase C and the three transferase A preparations were small, they were highly reproducible and reflect genuine differences in their degradative pathways. Secondly, Hayes *et al.* (1979) showed that transferase A and not transferase C is induced by phenobarbitone. If a post-translational modification were responsible for the conversion of transferase A into C, or vice versa, an intermediate (hybrid) form, which contained only one modified subunit, should be present in rat liver cytosol. This has not been found. Further, Habig *et al.* (1976) were unable to form hybrids of transferases A and C. This area clearly merits further investigation.

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References

- Bass, N. M., Kirsch, R. E., Tuff, S. A., Marks, I. & Saunders, S. J. (1977) *Biochem. Biophys. Acta* **492**, 163–175
- Bhargava, M. M., Listowsky, I. & Arias, I. M. (1978) *J. Biol. Chem.* **253**, 4116–4119
- Bhargava, M. M., Ohmi, N., Listowsky, I. & Arias, I. M. (1980) *J. Biol. Chem.* **253**, 718–723
- Calvert, R. & Gratzer, W. B. (1978) *FEBS Lett.* **86**, 247
- Carne, T., Tipping, E. & Ketterer, B. (1979) *Biochem. J.* **177**, 433–439
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
- Daniel, V., Smith, G. J. & Litwack, G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 1899–1902
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Fjellstedt, T. A., Allen, R. H., Duncan, B. K. & Jakoby, W. B. (1973) *J. Biol. Chem.* **248**, 3702–3707
- Grover, P. L. (1982) *Biochem. Soc. Trans.* **10**, 80–82
- Guthenberg, C. & Mannervik, B. (1979) *Biochem. Biophys. Res. Commun.* **86**, 1304–1310
- Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974a) *J. Biol. Chem.* **249**, 7130–7139
- Habig, W. H., Pabst, M. J., Fleischner, G., Gatmaitan, Z., Arias, I. M. & Jakoby, W. B. (1974b) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3879–3882
- Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1976) *Arch. Biochem. Biophys.* **175**, 710–716
- Hayes, J. D. (1980) Ph.D. Thesis, University of Edinburgh
- Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1979) *Biochem. J.* **181**, 699–708
- Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1980) *Biochem. J.* **185**, 83–87
- Hayes, J. D., Strange, R. C. & Percy-Robb, I. W. (1981) *Biochem. J.* **197**, 491–502
- Jakobson, I., Askelöf, P., Warholm, M. & Mannervik, B. (1977) *Eur. J. Biochem.* **77**, 253–262
- Jakoby, W. B. & Habig, W. H. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., ed.), vol. 11, pp. 63–94, Academic Press, London and New York
- Jakoby, W. B., Ketley, J. N. & Habig, W. H. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 213–220, Raven Press, New York
- Kalinyak, J. E. & Taylor, J. M. (1982) *J. Biol. Chem.* **257**, 523–530
- Kaplowitz, N., Percy-Robb, I. W. & Javitt, N. B. (1973) *J. Am. Med. Assoc.* **138**, 483–487
- Ketley, J. N., Habig, W. H. & Jakoby, W. B. (1975) *J. Biol. Chem.* **250**, 8670–8673
- Ketterer, B., Tipping, E., Beale, D. & Meuwissen, J. A. T. P. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 243–253, Raven Press, New York
- Ketterer, B., Carne, T. & Tipping, E. (1978) in *Transport by Proteins* (Blauer, G. & Sund, H., eds.), pp. 79–94, W. de Gruyter, Berlin and New York
- Ketterer, B., Beale, D. & Meyer, D. (1982) *Biochem. Soc. Trans.* **10**, 82–84
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Listowsky, I., Kamisaka, K., Ishitani, K. & Arias, I. M. (1976) in *Glutathione: Metabolism and Function* (Arias, I. M. & Jakoby, W. B., eds.), pp. 233–239, Raven Press, New York
- Litwack, G., Ketterer, B. & Arias, I. M. (1971) *Nature (London)* **234**, 466–467
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Pabst, M. J., Habig, W. H. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7140–7150
- Pattinson, N., Collins, D. & Campbell, B. (1980) *J. Chromatogr.* **187**, 409–412
- Scully, N. C. & Mantle, T. J. (1980) *Biochem. Soc. Trans.* **8**, 451–452
- Scully, N. C. & Mantle, T. J. (1981) *Biochem. J.* **193**, 367–370
- Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332