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Thiol–Protein Disulphide Oxidoreductases

DIFFERENCES BETWEEN PROTEIN DISULPHIDE-ISOMERASE AND GLUTATHIONE–INSULIN TRANSHYDROGENASE ACTIVITIES IN OX LIVER

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1. Protein disulphide-isomerase and glutathione–insulin transhydrogenase activities were assayed in parallel through a conventional purification of protein disulphide-isomerase from ox liver. 2. Throughout a series of purification steps (differential centrifugation, acetone extraction, $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography), the two activities appeared in the same fractions but were purified to different extents. 3. The final sample was 143-fold purified in protein disulphide-isomerase but only 10-fold purified in glutathione–insulin transhydrogenase; nevertheless the two activities in this preparation were not resolved by high-resolution isoelectric focusing and both showed pI 4.65. 4. In a partially purified preparation containing both activities, glutathione–insulin transhydrogenase was far more sensitive to heat denaturation than was protein disulphide-isomerase; conversely protein disulphide-isomerase was more sensitive to inactivation by deoxycholate. 5. The data are inconsistent with a single enzyme being responsible for all the protein disulphide-isomerase and glutathione–insulin transhydrogenase activity of ox liver. It is suggested that several similar thiol–protein disulphide oxidoreductases of overlapping specificities may better account for the data.

Protein disulphide-isomerase (EC 5.3.4.1) and glutathione–insulin transhydrogenase (EC 1.8.4.2, thiol–protein disulphide oxidoreductase) have both been detected in a wide range of mammalian tissues.

These thiol–protein disulphide transhydrogenases, and possibly others, may be involved in disulphide-bond formation and scission in protein synthesis and degradation, but their physiological role is not established, and important questions about the number, specificity and distribution of these activities remain unanswered. Protein disulphide-isomerase and GSH*–insulin transhydrogenase have both been purified from several sources and partially characterized (De Lorenzo *et al.*, 1966; De Lorenzo & Molea, 1967; Gurari, 1969; Varandani & Nafz, 1970; Ansorge *et al.*, 1973*a*,*b*; Varandani, 1974). The question of whether the two activities are in fact catalysed by a single enzyme (Katzen & Tietze, 1966; Varandani, 1974) has not been resolved, because few comparisons have been made directly in parallel experiments. Rather, investigations on subcellular distribution, substrate specificity, amino acid composition and molecular weight for each enzyme have been made on individual preparations, and the results compared with published data on the other enzyme, often derived by different methods, and often referring to a different species or tissue (Tomizawa & Varandani, 1965; Katzen & Tietze, 1966; Varandani & Nafz, 1970; Chandler & Varandani, 1972; Ansorge *et al.*, 1973*a*,*b*; Varandani, 1973, 1974). Moreover, the terms of the debate have been oversimplified by considering two possibilities only: (i) the two activities are catalysed by a single enzyme, or (ii) the activities are catalysed by two quite distinct enzymes; the possibility that there might be several similar enzyme species with rather different specificities has not been raised.

The most direct method of establishing the identity or difference of two similar enzyme activities is to study both activities in a crude source, and then to purify; even if the two activities are not clearly separable, they can be distinguished if the increase in specific activity of a purified sample compared with the starting material differs for the two activities. In this paper we adopt this approach and show differences in purification of the two activities throughout a standard purification of protein disulphide-isomerase from ox liver. This supplements the evidence of the preceding paper (Ibbetson & Freedman, 1976), showing that the membrane-bound protein disulphide-isomerase and GSH–insulin transhydro-

* Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

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genase activities of rat liver microsomal membranes are not catalysed by a single enzyme.

Experimental

Materials

Randomly reoxidized ribonuclease was obtained from Miles Laboratories Ltd., Slough, Bucks., U.K.; yeast RNA, myoglobin and ovalbumin were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; all other biochemicals were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

All chromatographic materials came from Pharmacia (G.B.) Ltd., London W.5, U.K.; Ampholines pH3.4–10 were from LKB Instruments Ltd., S. Croydon, Surrey, U.K.; Aristar acetone was from BDH Chemicals Ltd., Poole, Dorset, U.K.

All other reagents were AnalaR grade, obtained from BDH. Double-glass-distilled water was used throughout.

Preparation and purification of protein disulphide-isomerase

Protein disulphide-isomerase was prepared from ox liver by Gurari's (1969) modification of the standard procedure (De Lorenzo *et al.*, 1966). Liver obtained from a freshly slaughtered animal was divided into 1kg batches and stored at −20°C. Each batch was thawed slowly overnight at 4°C and all subsequent operations were carried out at 4°C. Liver (1kg) was chopped roughly into 2cm cubes free of connective tissue, washed twice in 1 litre of sucrose/Tris/EDTA buffer (10mM-Tris, containing 0.25M-sucrose and 5mM-EDTA, finally adjusted to pH7.8 with HCl), and then homogenized in 2 litres of the same buffer in 500ml batches, by using an Ato-Mix blender operated at top speed for 1min. The combined homogenates were centrifuged at 1350g for 20min to sediment nuclei and whole cells (pellet 1), and the combined supernatants were centrifuged at 14000g for 20min to sediment mitochondria and lysosomes (pellet 2). Combined supernatants from the second spin were stirred gently, and adjusted slowly to pH5.2 by dropwise addition of approx. 30ml of 2M-acetic acid. Stirring was then continued for a further 30min while the microsomal material aggregated. Centrifugation at 15000g for 20min sedimented the microsomal fraction (pellet 3), which was then taken up in the minimum volume of 10mM-Tris/HCl buffer, pH7.8; this suspension was homogenized gently by hand to give approx. 300ml of a thick light-brown slurry, which was stored at −20°C overnight before further use.

The microsomal slurry was thawed and then added slowly with vigorous stirring to 10vol. of Aristar acetone previously chilled to −15°C. The resulting suspension was filtered at the pump by using a Buchner funnel and Whatman no. 54 (hardened) filter paper. The buff-coloured acetone-dried powder was washed several times with further cold Aristar acetone until the filtrate was colourless, and was then dried under vacuum overnight until it became a very pale sandy colour.

The acetone-dried powder was then extracted twice in 10mM-Tris/HCl buffer, pH7.8. It was gently hand-homogenized in 8–10ml of buffer/g, stirred gently for 2h, and centrifuged at 78000$g_{av.}$ for 20min. The supernatant was retained, and the pellets were re-extracted in 2–3ml of buffer/g as before. The supernatants were dialysed against 10–20vol. of the same buffer for 48h, with one change of buffer.

The combined dialysed extract was partially purified by $(NH_4)_2SO_4$ precipitation. It was stirred gently while being adjusted to 50% saturation by the gradual addition over 1h of solid $(NH_4)_2SO_4$ (0.25g/ml). Stirring was continued a further 1h, and the suspension was then centrifuged at 14000g for 20min. The red–gold supernatant was adjusted to saturation by the addition of further $(NH_4)_2SO_4$ (0.36g/ml) and the resultant suspension was treated as before. The pinkish pellet from the second centrifugation was dissolved in approx. 100ml of 20mM-KH_2PO_4/Na_2HPO_4 buffer, pH6.3, and dialysed against 10–20vol. of the same buffer over 48h, with one change of buffer.

The red pigment was removed by ion-exchange chromatography on a column (5cm×30cm) of CM-Sephadex C-50, previously equilibrated in the phosphate buffer. Batches of the dialysed protein (50ml, approx. 700mg of protein) were eluted with the same buffer, leaving red impurities at the top of the column. Fractions with isomerase activity were combined, and the pale-yellow solution was concentrated to one-fifth of its original volume by pressure dialysis (ultrafiltration cell from Chemlab Instruments, Hornchurch, Essex, U.K., containing a Diaflo Ultrafilter PM10 membrane with cut-off mol.wt. 10000).

The solution was dialysed for 24h against 50vol. of 0.1M-Tris/HCl buffer, pH7.8, and was then eluted on a column (2cm×35cm) of DEAE-Sephadex A-50, previously equilibrated in the same buffer. A linear NaCl gradient was applied (0–0.7M-NaCl) with 500ml in each reservoir. Combined fractions with isomerase activity were concentrated to one-quarter of their original volume by pressure dialysis, and the pale-cream solution was dialysed against 100vol. of 50mM-$(NH_4)_2CO_3$ for 48h with one change of buffer before freeze-drying.

The freeze-dried material was dissolved in a small volume of 20mM-phosphate buffer, pH6.3, and eluted on a column (1cm×24cm) of DEAE-Sephadex A-50, previously equilibrated in the buffer. A linear NaCl gradient was applied (0.1–0.7M-NaCl) with 250ml in each reservoir. Fractions with isomerase activity were combined, concentrated by

pressure dialysis and dialysed against 50mM-$(NH_4)_2CO_3$, and the colourless solution was stored at −20°C for subsequent analysis.

Assays for protein

Protein was assayed as a routine by the Folin–Ciocalteu method, with bovine serum albumin (fraction V) as standard (Lowry *et al.*, 1951). Protein samples from isoelectric-focusing experiments were assayed with Eosin, by using a modification (Peters *et al.*, 1972) of the original procedure (Hiraoka & Glick, 1963).

Enzyme assays

Glutathione–insulin transhydrogenase was assayed by a spectrophotometric method using NADPH and glutathione reductase to reduce GSSG: the procedure is described in the preceding paper (Ibbetson & Freedman, 1976). The buffer used in most cases was 10mM-Tris/HCl, pH7.8, containing 5mM-EDTA; exceptions were: (i) for assays of samples that were very dilute and in phosphate buffer, pH6.3, the Tris/HCl concentration in the assay buffer was increased to 0.1M; (ii) for assays of membrane fractions, the buffer contained 0.25M-sucrose and was flushed with CO for 20min immediately before use to minimize endogenous NADPH oxidation (Ibbetson & Freedman, 1976). Enzyme activity was monitored at 30°C by following the rate of oxidation of NADPH at 340nm. Controls are described in the preceding paper (Ibbetson & Freedman, 1976); 1 unit is defined as an amount of enzyme catalysing the formation of 1 μmol of GSSG/min.

Protein disulphide-isomerase was assayed by the re-activation of randomly reoxidized ribonuclease; the method is described in the preceding paper (Ibbetson & Freedman, 1976). Samples were incubated with the substrate and dithiothreitol at 30°C in the same buffers used for the transhydrogenase assays. Assays of ribonuclease activity were all carried out in 10mM-Tris/HCl/5mM-EDTA, pH7.8 at 30°C, with high-molecular-weight RNA as substrate; activity was measured by dual-wavelength spectrophotometry by following the rate of change of E_{260} relative to the E_{280}, and 1 unit of ribonuclease activity is here defined as that producing a relative absorbance change of 1 unit/min. One unit of isomerase activity is then defined as that which activates randomly reoxidized ribonuclease at the rate of 1 ribonuclease unit/min.

Isoelectric focusing

Gels (65mm×5mm) containing 7.5% (w/v) acrylamide, 1% (w/v) ampholyte pH3.5–10 and 200–250 μg of protein, were polymerized with persulphate. Electrophoresis was carried out at 2mA/gel at 4°C (Shandon electrophoresis apparatus and Vokam power pack), by the method of Righetti & Drysdale (1971). After ampholyte removal in 5% (v/v) trichloroacetic acid, gels were stained with Amido Black (Wrigley, 1971) or Coomassie Brilliant Blue (Weber & Osborn, 1969), de-stained electrophoretically, and stored in 7% (v/v) acetic acid. Gels containing no protein were cut into 10 or 20 sections, macerated in 7vol. of 10mM-NaCl, and left at room temperature overnight; pH measurements were made at room temperature on a Pye–Unicam 290 pH-meter with expanded scale, by using a Russell combination pH electrode.

Small-range ampholytes were prepared by electrophoresis of gels, containing 8% (w/v) ampholyte pH3.5–10, at 4°C for 2.5h. Those sections of the gels corresponding to pH4–6 were macerated in an equal volume of water and left at room temperature overnight. After filtering at a water pump the filtrate was diluted 1:1 (v/v) in subsequent gels.

Protein and enzyme activities were assayed on the supernatant from gel sections macerated in 0.5ml of 0.1M-Tris/HCl/5mM-EDTA, pH7.8, and left overnight at 4°C.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The procedure of Weber & Osborn (1969) was followed on gels (65mm×5mm) containing 10% (w/v) acrylamide and 0.1% (w/v) sodium dodecyl sulphate; markers were cytochrome *c*, myoglobin, trypsin, carboxypeptidase, ovalbumin and bovine serum albumin. Protein samples were incubated in 10mM-KH_2PO_4/Na_2HPO_4 buffer, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (w/v) β-mercaptoethanol at 37°C for 2.5h, and then dialysed against the same buffer containing 0.1% sodium dodecyl sulphate and 0.1% β-mercaptoethanol at 25°C overnight. Gels were stained with Coomassie Brilliant Blue, de-stained electrophoretically, and stored in 7% (v/v) acetic acid. Chromoscans were obtained with a Joyce–Loebl gel scanner, by using a 575nm filter.

For subsequent analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, gel sections after isoelectric focusing were incubated in 125 μl of 10mM-phosphate buffer, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (w/v) β-mercaptoethanol at 25°C overnight without maceration. Electrophoresis required 50 μl of undialysed supernatant/gel.

Results and Discussion

Distribution of isomerase and transhydrogenase in ox liver homogenate

Ox liver was homogenized and then fractionated by three successive centrifugations (see the Experimental section). Both isomerase and transhydrogenase activities were detected in all three pellets and

Table 1. *Distribution of isomerase and transhydrogenase activities in fractions from an ox liver homogenate*

The homogenate from 1 kg of ox liver in sucrose/Tris/EDTA buffer, pH 7.8 (10mM-Tris/HCl containing 0.25M-sucrose and 5mM-EDTA), was fractionated by three successive centrifugations at 4°C, and the pellets were re-homogenized in the same medium. Pellet 1, 'nuclear', 1350*g* for 20min; pellet 2, 'mitochondrial', 14000*g* for 20min; pellet 3, 'microsomal', 15000*g* for 20min after acidification to pH 5.2. Enzyme activities were measured at pH 7.8 at 30°C; transhydrogenase assays were performed in the absence of O_2 by flushing the system with CO. Details are given in the text.

	A: Total recovered			B: Distribution (as % of total recovered)			C: Specific activity (units/g of protein)	
Fraction	Protein (g)	Isomerase (units)	Trans-hydrogenase (units)	Protein	Isomerase	Trans-hydrogenase	Isomerase	Trans-hydrogenase
Homogenate	132.2	508	128.0	—	—	—	3.8	1.0
Pellet 1	24.8	85	17.6	19	19	17	3.4	0.7
Pellet 2	54.6	158	30.8	41	36	29	2.9	0.6
Pellet 3	21.9	137	28.9	16	31	27	6.3	1.3
Supernatant 3	31.9	64	28.3	24	14	27	2.0	0.9

Table 2. *Recovery of isomerase and transhydrogenase activities in solution after lipid extraction of microsomal membranes*

Lipid was extracted from the resuspended microsomal pellet by acetone at −15°C, and the residual powder was dried. The powder was extracted twice with 10mM-Tris/HCl, pH 7.8, at 4°C throughout, the combined extracts were dialysed, and the pellets were re-homogenized. Enzyme activities were measured at pH 7.8 at 30°C. Details are given in the text.

	Total recovered			Specific activity (units/g of protein)	
Fraction	Protein (mg)	Isomerase (units)	Transhydrogenase (units)	Isomerase	Transhydrogenase
Microsomal suspension	21900	123	35.7	5.6	1.6
Combined extract	4340	128	15.2	29.5	3.5
Final pellets	11000	12	0.5	1.1	0.05

in the supernatant (Table 1, A) but the distributions of the two activities were different (Table 1, B). Both were present at highest specific activity in pellet 3 (microsomal pellet), but, whereas for isomerase this had a specific activity 1.7-fold that of the homogenate, the increase for transhydrogenase was 1.3-fold (Table 1, C). This is mainly because of the higher proportion of transhydrogenase activity appearing in the supernatant fraction. The large amounts of both activities in pellet 2, and the consequent low recoveries in pellet 3, are the result of the vigorous second centrifugation, which was intended to minimize lysosomal contamination of pellet 3, the 'microsomal' fraction. Pellet 3 is obtained by acidifying the post-lysosomal supernatant and then spinning for 20min at 15000*g*, so, although the high yield of GSH–insulin transhydrogenase in the final supernatant may reflect a cytosol origin for part of the transhydrogenase activity, it might be the result of solubilization of this activity in acid conditions. Without marker-enzyme studies no significant conclusions can be drawn about the subcellular distributions of the two activities, but the difference in distribution between them is significant. The overall yield of both activities in the four fractions was quite high, 87% for isomerase and 83% for transhydrogenase.

Extraction and purification of protein disulphide-isomerase

Protein disulphide-isomerase was extracted from pellet 3, and purified by published procedures; isomerase and GSH–insulin transhydrogenase activities were monitored in all fractions at each step.

Lipid was extracted from the resuspended microsomal slurry by cold acetone, and then soluble protein was extracted from the acetone-dried powder. Both activities were present in the extract; neither was retained in the residue (Table 2). But the effective purifications of the two activities were quite different. The overall yield of protein in extracts and pellet was 70%, whereas the yield for isomerase was 114% and for transhydrogenase was 44%. In view of these marked differences it is likely that solubilization leads to a genuine activation of isomerase but an inactivation of transhydrogenase. Inactivation of GSH–insulin transhydrogenase by acetone has been reported by Ansorge *et al.* (1973*a*). The acetone-drying and extraction gave an apparent 5.3-fold purification of isomerase, but

only 2.2-fold purification of transhydrogenase (Table 2).

The extract was subsequently purified by $(NH_4)_2SO_4$ precipitation followed by ion-exchange chromatography. The protein precipitated at 50% saturation contained negligible isomerase and transhydrogenase activities, but both were present in the 50–100%-satd. precipitate; the isomerase was slightly purified by this procedure, but the transhydrogenase was not (Table 3). As with the solubilization, this step in purification of protein disulphide-isomerase did not separate it from GSH–insulin transhydrogenase, but caused a net loss of transhydrogenase activity. This suggests that species with GSH–insulin transhydrogenase activity, distinct from those with protein disulphide-isomerase activity, are inactivated in the course of purification.

Fractions eluted from the chromatography columns were assayed individually for the two activities. Elution on CM-Sephadex at pH 6.3 yielded one broad peak of protein with coincident peaks of both isomerase and transhydrogenase activities (results not shown). Elution of the pooled material on DEAE-Sephadex at pH 7.8 showed the presence of four main protein bands (Fig. 1). Isomerase and transhydrogenase activities were mainly associated with the third component and showed similar elution profiles. The material before this purification step also showed GSSG–NADPH oxidoreductase and 5,5′-dithiobis-(2-nitrobenzoic acid)–NADPH oxidoreductase activities (Eriksson *et al.*, 1974); their elution profiles on this second column were distinct both from isomerase and transhydrogenase and from each other (results not shown). The co-elution of isomerase and transhydrogenase activities from these two columns might be interpreted to mean that at this stage a single enzyme species is responsible for both activities; however, the increases in specific activity of the two activities were again different (Table 3). In an attempt to solve the problem, fractions 48–59, containing both isomerase and transhydrogenase activities, were pooled and freeze-dried, and various treatments were applied to this material to determine their effects on the two activities.

Effects of heat and deoxycholate on the partially purified material

The material was dissolved in 10mM-Tris/HCl, pH 7.8, containing 5mM-EDTA; samples were incubated in a water bath at 50° or 90°C for various periods and were then kept on ice until assayed. The two activities responded differently to heat, with

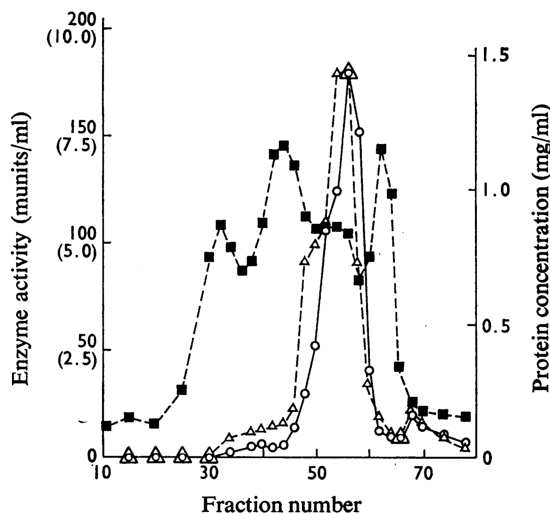

Fig. 1. *Elution profiles of isomerase and transhydrogenase activities after chromatography on DEAE-Sephadex, pH 7.8*

A column (35cm×2cm) of DEAE-Sephadex was equilibrated in 0.1M-Tris/HCl, pH 7.8, at 4°C; 670mg of protein in 32ml was applied and eluted with a linear gradient of 0–0.7M-NaCl in 1 litre of the same buffer. Fractions (10ml) were collected at a rate of 25ml/h, and assayed for protein (■), isomerase activity (○ on scale 0–200), and transhydrogenase activity (△ on scale 0–10). Fractions 48–59 were combined for subsequent treatment.

Table 3. *Purification of solubilized isomerase and transhydrogenase activities*

Details of the purification procedure, carried out entirely at 4°C, are given in the text; enzyme activities were measured at pH 7.8 at 30°C.

Purification step	Total recovered: Protein (mg)	Total recovered: Isomerase (units)	Total recovered: Transhydrogenase (units)	Specific activity (units/g of protein): Isomerase	Specific activity (units/g of protein): Transhydrogenase
Combined extract	4340	128	15.2	29.5	3.5
0–50%-satd. $(NH_4)_2SO_4$	1190	12	0.40	10.3	0.3
50–100%-satd. $(NH_4)_2SO_4$	2370	90	5.64	38.0	2.4
CM-Sephadex, pH 6.3	670	43	1.67	64.2	2.5
DEAE-Sephadex, pH 7.8	150	20	1.13	133	7.5
DEAE-Sephadex, pH 6.3	35	19	0.35	543	10.0

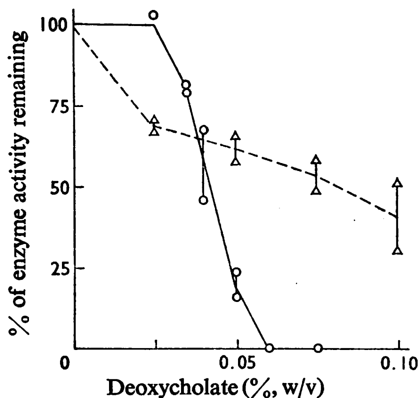

Fig. 2. *Effect of deoxycholate treatment on isomerase and transhydrogenase activities*

Freeze-dried protein from DEAE-Sephadex column, pH7.8, was redissolved in 10mM-Tris/HCl/5mM-EDTA, pH7.8, and assayed for isomerase (○) and transhydrogenase (△) activities in the presence of sodium deoxycholate at 30°C. Results of duplicate experiments are shown.

isomerase the more stable under all conditions. For example, after 5min at 50°C there was a 5% loss of isomerase activity but a 23% loss of transhydrogenase activity; after 5min at 90°C there was 51% loss of isomerase activity but 80% loss of transhydrogenase activity.

The isomerase and transhydrogenase activities of this preparation also showed different responses to the presence of sodium deoxycholate in enzyme assays at 30°C. Transhydrogenase showed a gradual decline in activity with deoxycholate concentration (Fig. 2), but considerable activity was still detectable at 0.1% (w/v) deoxycholate, whereas isomerase was inactivated completely in the range between 0.03 and 0.06% deoxycholate. The maximum concentration of deoxycholate (0.1%) did not affect ribonuclease activity in the isomerase assay nor GSSG–NADPH oxidoreductase in the transhydrogenase assay.

These findings on the inhibition by deoxycholate of the soluble enzyme activities are comparable with those for membrane-bound activities in the preceding paper (Ibbetson & Freedman, 1976); it was shown that, in rat liver microsomal fractions, protein disulphide-isomerase is far more susceptible to inhibition by the detergent than is GSH–insulin transhydrogenase.

Attempt at further purification

These differences in stability implied that the two activities were catalysed by distinct species which might be separable, but a further ion-exchange-chromatographic step on DEAE-Sephadex at pH6.3 (the final step of the published purification of isomerase) gave no separation (Fig. 3). The elution gave a single peak of protein containing no reductase activities, and with corresponding profiles for both isomerase and transhydrogenase. However, although 95% of the applied isomerase activity was recovered in this elution the yield of transhydrogenase in this step was only 31% (Table 3). Thus, at every stage in the purification from the ox liver homogenate, isomerase and transhydrogenase are most concentrated in the same fractions, but the degrees of purification are different at each step. The purification data summarized in Table 4 show that, after the third ion-exchange column, isomerase is 143-fold purified overall, whereas transhydrogenase is only 10-fold purified. But at no stage has transhydrogenase activity been detected in any of the discarded fractions.

Co-purification of two activities with a constant ratio of specific activities is a criterion commonly used to establish that two activities are functions of the same enzyme. Thus Hayakawa *et al.* (1975) examined the GSH *S*-epoxidetransferase activities of a sheep

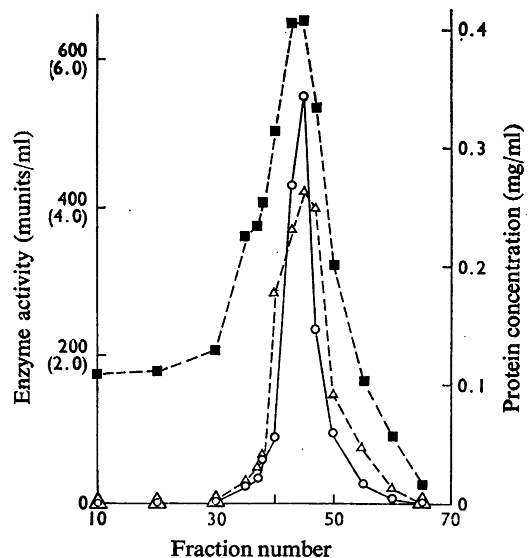

Fig. 3. *Elution profiles of isomerase and transhydrogenase activities after chromatography on DEAE-Sephadex, pH6.3*

A column (25cm×1cm) of DEAE-Sephadex was equilibrated in 20mM-phosphate buffer, pH6.3, at 4°C; 55mg of protein in 2.5ml was applied and eluted with a linear gradient of 0.1–0.7M-NaCl in 500ml of the same buffer. Fractions (5ml) were collected at a rate of 10ml/h, and assayed for protein (■), isomerase activity (○ on scale 0–600), and transhydrogenase activity (△ on scale 0–6). Fractions 38–55 were combined for subsequent treatment.

Table 4. *Summary of purification of protein disulphide-isomerase*

Data for the homogenate and pellet 3 are derived from Table 1 and the remainder from Table 3.

Fraction	Purification	
	Isomerase	Trans-hydrogenase
Homogenate	1.0	1.0
Pellet 3	1.7	1.3
Acetone-dried powder extract	7.8	3.5
50–100%-satd. $(NH_4)_2SO_4$	10.0	2.4
CM-Sephadex, pH 6.3	16.9	2.5
DEAE-Sephadex, pH 7.8	35.1	7.5
DEAE-Sephadex, pH 6.3	143	10.0

liver homogenate and of a purified sample on over 50 epoxide substrates. The two preparations showed the same pattern of substrate preferences; for all the substrates the purified sample showed the same increase in specific activity, and it was concluded that a single enzyme could account for all the GSH *S*-epoxidetransferase activity, in contrast with the situation in the rat and the guinea pig (Habig *et al.*, 1974; Oesch *et al.*, 1971). Using the same criterion, Ondarza *et al.* (1974) established that in rat liver the NADPH-dependent reduction of the mixed disulphide of CoA and GSH was catalysed by the conventional GSSG–NADPH oxidoreductase; differences in degree of purification for the 5,5′-dithiobis-(2-nitrobenzoic acid)–NADPH oxidoreductase activity established that it was not catalysed by the same enzyme. For protein disulphide-isomerase and glutathione–insulin transhydrogenase studied in the present paper, the differences in purification at each step are clearly inconsistent with their being catalysed by a single enzyme species.

The heterogeneity and other properties of the final enzymically active material were studied by isoelectric focusing and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Focusing using fractionated ampholytes to give high resolution in the range pH 4–6 showed the presence of five bands very close together in the range pH 4.5–4.8. Other gels focused under the same conditions (4°C, 5h) were not stained but were sectioned and analysed for protein contents and enzyme activities (Fig. 4). Isomerase and transhydrogenase activities were not resolved by this treatment; both showed maximum activity in a section corresponding to pH 4.65. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified material showed two discrete peaks corresponding to material of mol.wt. 63500 and 58000 respectively, a smaller discrete peak corresponding to material of mol.wt. 48500 and a more diffuse band at about mol.wt. 30000. Material that had been

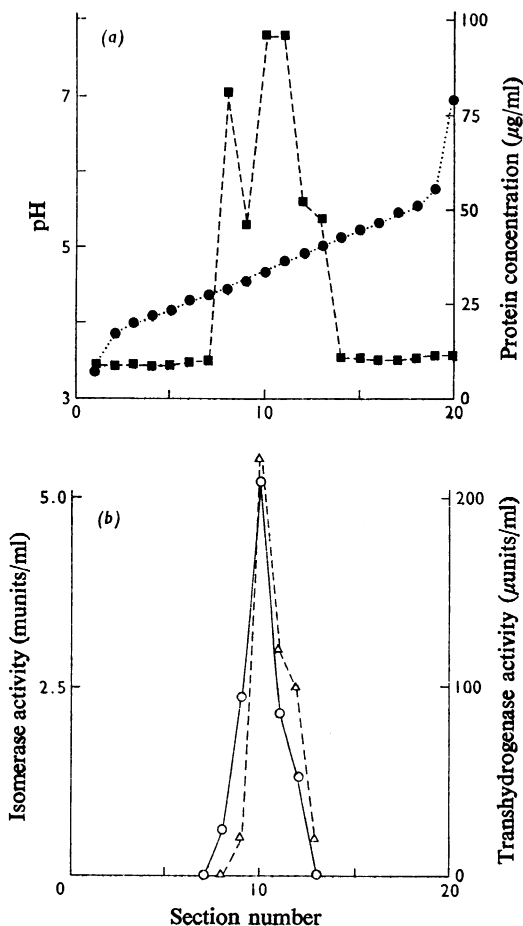

Fig. 4. *Isoelectric focusing by polyacrylamide-gel electrophoresis*

Electrophoresis of purified protein was carried out at 2mA/gel at 4°C for 5h on gels (65mm×5mm) containing 7.5% (w/v) acrylamide and 250μg of protein. The pH gradient (●) was measured at room temperature after gel sections (3.3mm×5mm) were macerated in 10mM-NaCl and left overnight at room temperature. Other sections were macerated in 0.1M-Tris/HCl/5mM-EDTA, pH 7.8, and left overnight at 4°C; the supernatant was assayed for protein (■), isomerase activity (○) and transhydrogenase activity (△).

subjected to isoelectric focusing was also studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; each of the active fractions 8–13 from the focused gel (Fig. 4) showed two discrete peaks of material with average mol.wts. 65000 and 57500, corresponding to the major peaks in the unfocused material. (This might suggest that complete isoelectric focusing had not been achieved, but this is unlikely,

since the run was for 5h; in any case a longer period of focusing led to considerable inactivation of the two activities.)

Conclusion

Several findings in this paper are incompatible with the view that protein disulphide-isomerase and GSH–insulin transhydrogenase activities in ox liver can be accounted for by a single enzyme species: (i) the distributions of the two activities in a homogenate are different, with a significantly higher proportion of transhydrogenase activity remaining in the final supernatant; (ii) on solubilization of the activities from microsomal membranes by buffer extraction of an acetone-dried powder, there is a net activation of protein disulphide isomerase but considerable loss of GSH–insulin transhydrogenase activity; (iii) at every stage in the subsequent purification of protein disulphide-isomerase from this extract, the degree of purification of transhydrogenase is different from that of isomerase, so that a preparation which is purified 143-fold in isomerase activity compared with the homogenate is only 10-fold purified in transhydrogenase activity; (iv) in a partially purified sample showing both activities, GSH–insulin transhydrogenase is more sensitive to heat inactivation than is protein disulphide-isomerase; conversely, protein disulphide-isomerase is completely inhibited by deoxycholate at concentrations above 0.06%, whereas inhibition of transhydrogenase is far less marked.

To maintain the hypothesis of a single enzyme, one would have to disregard the findings on subcellular distribution and propose either (*a*) that the active site shows a selective instability, so that heat treatment and most purification procedures decrease its activity only in the glutathione–insulin transhydrogenase reaction, whereas deoxycholate selectively inhibits the activity towards randomly reoxidized ribonuclease, or (*b*) that the enzyme contains multiple active sites, all catalysing thiol/disulphide interchange, but differing in specificity and stability. Neither proposal is plausible and neither retains the simplicity that is the one real virtue of the 'single enzyme' hypothesis.

Although ruling out the possibility of a single enzyme being entirely responsible for both activities, these experiments have not identified two distinct enzyme species. The failure to separate isomerase from transhydrogenase activity, their co-elution at each stage and their very similar distribution in a high-resolution isoelectric-focusing gel suggest that very similar proteins may be involved. Further, samples of isomerase and GSH–insulin transhydrogenase that have been purified to homogeneity act on a considerable range of disulphide substrates (Steiner *et al.*, 1965; Varandani *et al.*, 1975). In view of this broad specificity, it is possible that several proteins, each with thiol–protein disulphide oxidoreductase activity but with different substrate preferences, all contribute to the total protein disulphide-isomerase and glutathione–insulin transhydrogenase activity of ox liver. This alternative would explain the apparent contradiction between the differences in behaviour of the two activities in homogenates and impure samples, and their apparent coexistence in samples purified to homogeneity. There are precedents for this suggestion; overlapping substrate specificities have been demonstrated for four distinct glutathione *S*-transferases from rat liver, which are almost identical in molecular weight but separable by ion-exchange chromatography (Habig *et al.*, 1974). It is also now known that the synthesis of several different forms of rat liver microsomal cytochrome *P*-450 with overlapping substrate specificities can be induced (Lu *et al.*, 1973; Fujita *et al.*, 1973). Studies with a range of thiol and disulphide substrates on crude homogenates and on samples purified to homogeneity will be necessary to establish if such a multiplicity of enzyme species also occurs in thiol–protein disulphide oxidoreductases.

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