Resolution of protein disulphide-isomerase and glutathione–insulin transhydrogenase activities by covalent chromatography

Properties of the purified protein disulphide-isomerase

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1. Protein disulphide-isomerase (EC 5.3.4.1) and glutathione–insulin transhydrogenase (EC 1.8.4.2) were resolved by covalent chromatography. Both activities, in a partially purified preparation from bovine liver, bind covalently as mixed disulphides to activated thiopropyl-Sephadex 6B, but in a new stepwise elution procedure protein disulphide-isomerase is displaced in mildly reducing conditions whereas glutathione–insulin transhydrogenase is only displaced by more extreme reducing conditions. 2. This, together with evidence for partial resolution of the two activities by ion-exchange chromatography, conclusively establishes that the two activities are not alternative activities of a single bovine liver enzyme. 3. Protein disulphide-isomerase, partially purified by a published procedure, has now been further purified by covalent chromatography and ion-exchange chromatography. The final material is 560-fold purified relative to a bovine liver homogenate; it has barely detectable glutathione–insulin transhydrogenase activity. 4. The purified protein disulphide-isomerase shows a single major band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis corresponding to a mol.wt. of 57000. 5. The purified protein disulphide-isomerase has K_m values for ‘scrambled’ ribonuclease and dithiothreitol of 23 $\mu\text{g}/\text{ml}$ and 5.4 μM respectively and has a sharp pH optimum at 7.5. The enzyme has a broad thiol-specificity, and several monothiols, at 1 mM, can replace dithiothreitol. 6. The purified protein disulphide-isomerase is completely inactivated after incubation with a 2–3-fold molar excess of iodoacetate. The enzyme is also significantly inhibited by low concentrations of Cd^{2+} ions. These findings strongly suggest the existence of a vicinal dithiol group essential for enzyme activity. 7. When a range of thiols were used as co-substrates for protein disulphide-isomerase activity, the activities were found to co-purify quantitatively, implying the presence of a single protein disulphide-isomerase of broad thiol-specificity. Glutathione–disulphide transhydrogenase activities, assayed with a range of disulphide compounds, did not co-purify quantitatively.

Several physiological processes are known to involve reactions between thiol groups and disulphide bonds of proteins. Such thiol–protein disulphide interchange reactions or thiol–protein disulphide oxidoreductions are involved in the degradation of polypeptide hormones, in the assembly of many multi-chain proteins, in the regulation of enzyme activity through intercon-

version of enzymes between alternative forms and in the process of formation of native disulphide bonds during the folding of reduced proteins (for review see Freedman, 1979). The enzymology of such processes is very little understood, and few enzymes catalysing these reactions have been characterized in any detail. Two enzyme activities that have been extensively studied are protein disulphide-isomerase (EC 5.3.4.1) and glutathione–insulin transhydrogenase (EC 1.8.4.2, sometimes known as thiol–protein disulphide oxidoreductase). The former is defined by its thiol-dependent catalysis of the rearrangement of non-native disulphide bonds in ‘randomly’ reoxidized ribonuclease, leading to the

Abbreviations used: RNAase, ribonuclease; SDS, sodium dodecyl sulphate; GSH and GSSG, reduced and oxidized glutathione.

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formation of the native structure and activity. The latter is defined by the reduction of the disulphide bonds of insulin by a thiol such as GSH, a process that is probably important in insulin catabolism.

The relationship between these two enzyme activities has been controversial for some time. Similarities between the properties of purified preparations of the two activities led to the suggestion that a single enzyme was involved, the two activities being alternative expressions of the activity of a single protein species (Ansorge *et al.*, 1973*b*; Varandani, 1974, 1978). However, when the two activities are studied in parallel, clear differences are apparent. In bovine and rat liver microsomal fractions, and in solubilized preparations derived from them, the two activities respond differently to treatment with heat, detergents and organic solvents (Ibbetson & Freedman, 1976; Hawkins & Freedman, 1976; Freedman *et al.*, 1978). When assayed in parallel, the two activities were found to have different tissue and subcellular distributions in sheep tissues (Hillson, 1979). Furthermore, the activities do not co-purify quantitatively (Hawkins & Freedman, 1976). These findings strongly suggest that separate enzymes are responsible for the two activities, but until now the enzymes have not been resolved (Hawkins & Freedman, 1976).

We have now investigated alternative separative methods and have developed a new application of covalent chromatography that reproducibly resolves protein disulphide-isomerase and glutathione-insulin transhydrogenase activities from bovine liver. The procedure depends on specific immobilization to the gel matrix of proteins containing reactive thiol groups, and exploits the suggestion from earlier work (DeLorenzo *et al.*, 1966*a,b*; Varandani & Plumley, 1968) that the enzymes responsible for the two activities in question both have essential thiol groups. Thiol-containing proteins can be bound covalently as mixed disulphides to activated thiopropyl-Sepharose 6B gel. The bound proteins can then themselves be displaced by treatment with excess of a simple thiol. In the past this technique has been used mainly for separating proteins or peptides containing the thiol group from those lacking this group; the former are retained on the gel whereas the latter pass through unbound. In the present paper we describe our extension of this approach to resolve distinct thiol-containing proteins by immobilizing both on the gel and then using a series of reductive elutions to displace them in turn. This successfully resolves the two activities in material partially purified from bovine liver; it demonstrates convincingly that the two activities are not catalysed by a single enzyme species. We also describe some properties of the purified protein disulphide-isomerase thereby obtained, and some experiments that lend support to the view that bovine

liver contains multiple enzymes capable of catalysing the general thiol-protein disulphide oxidation-reduction reaction. A preliminary account of some of this work has already been published (Hillson & Freedman, 1979).

Experimental

Materials

The following were supplied by Sigma (London) Chemical Co. (Poole, Dorset, U.K.): bovine serum albumin (fraction V), L-cysteine hydrochloride, L-cystine, 2':3'-cyclic CMP, 5,5'-dithiobis-(2-nitrobenzoic acid), 2,2'-dithiopyridine, DL-dithiothreitol, GSH, glutathione reductase (type III, yeast), insulin (bovine pancreas), iodoacetic acid, 2-mercaptoethanol (type I), NADPH (tetrasodium salt, type III), ribonuclease A (type III-A, bovine pancreas), thioglycolic acid (2-mercaptoacetic acid; grade IV), *trans*-4,5-dihydroxy-1,2-dithiane (oxidized dithiothreitol), Triton X-100 and Tris base.

Koch-Light Laboratories (Colnbrook, Bucks., U.K.) supplied highly polymerized yeast RNA (sodium salt). Miles-Seravac (Maidenhead, Berks., U.K.) supplied randomly reoxidized ('scrambled') RNAase. Materials for gel filtration, ion-exchange chromatography and thiopropyl-Sepharose 6B for covalent chromatography were supplied by Pharmacia (G.B.) (London W.5, U.K.). All other materials were A.R. grade where possible and were supplied by BDH Chemicals (Poole, Dorset, U.K.) or Fisons Scientific Apparatus (Loughborough, Leics., U.K.).

Purification of protein disulphide-isomerase

Partial purification. The enzyme was purified from bovine liver by methods based on those of Hawkins & Freedman (1976). The basic procedure, involving homogenization, isolation of the microsomal fraction, extraction with acetone, solubilization of the enzyme, $(\text{NH}_4)_2\text{SO}_4$ precipitation, ion-exchange chromatography on CM-Sephadex C-50 at pH 6.3 and on DEAE-Sephadex at pH 7.8, was performed as described previously (Hawkins & Freedman, 1976). The resulting material was dialysed against 50 mM- NH_4HCO_3 , pH 7.8, and freeze-dried; the stored powder, designated 'DEAE pH 7.8 eluate', was the starting point for the further purification procedures described below.

Ion-exchange chromatography. For further purification by ion-exchange chromatography, the DEAE pH 7.8 eluate was dissolved in 20 mM- $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.3, at a concentration of 20 mg/ml and applied to a column (1 cm $\times$ 30 cm) of DEAE-Sephadex A-50 previously equilibrated in the same phosphate buffer. The material was eluted at 4°C with increasing concentrations of NaCl in the same buffer. Various

patterns of elution were used, and the profile of the salt gradient in eluted fractions was determined from measurements of refractive index. A similar procedure was followed for the final purification of fractions obtained from covalent chromatography (see the legend to Fig. 1).

Covalent chromatography. The standard buffer for covalent chromatography was 50 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl, 5 mM-MgCl₂, 1.25 mM-EDTA and 0.1 M-NaCl (TKM/EDTA/NaCl buffer). Samples for covalent chromatography were dissolved in 2–10 ml of this buffer to a protein concentration of 10 mg/ml and were gently reduced by reaction with 0.1 mM-dithiothreitol for 30 min at 30°C, followed by desalting on Sephadex G-25 with TKM/EDTA/NaCl buffer. Thiopropyl-Sepharose 6B was used in the 'activated' mixed-disulphide form, and was swollen and washed free of additives in TKM/EDTA/NaCl buffer (200 ml/g of freeze-dried gel), and then equilibrated in this buffer at room temperature (approx. 20°C). Reduced protein was coupled to the gel either by loading on to a column of thiopropyl-Sepharose 6B followed by incubation of the column at room temperature for 30–60 min, or by incubation of the reduced protein and gel at 25°C for 16 h with gentle shaking, before the column was poured. The latter procedure was used in preparative-scale experiments. In analytical experiments, 10 g wet wt. of gel was used (1 cm × 10 cm column); in preparative experiments, 30–45 g wet wt. of gel was used.

After the sample was loaded, the column was equilibrated to 4°C, and unbound protein was eluted with TKM/EDTA/NaCl buffer. Bound proteins were then displaced from the gel by successive elutions with solutions containing low-molecular-weight thiols in order of increasing reducing power, namely 20 mM-L-cysteine, 50 mM-GSH and 20 mM-dithiothreitol, each in TKM/EDTA/NaCl buffer. For each elution step, 1 void volume of eluent was run into the column and the column was then incubated at 30°C for 30 min to allow reaction to occur. Elution was then continued at 4°C at flow rates of 2–10 ml/h, and 2 ml fractions (analytical scale) or 5 ml fractions (preparative scale) were collected. A wash step with TKM/EDTA/NaCl buffer alone was included after each thiol-containing step. Fractions were monitored for protein at 280 nm and for displaced thiopyridone at 343 nm.

For each step, fractions containing protein were pooled and treated with 5 mM-dithiothreitol for 30 min at 25°C (to reduce any mixed disulphides between protein and eluent), then dialysed extensively against TKM buffer, pH 7.5, at 4°C. The procedure resulted in the preparation of four fractions, namely: unbound protein (fraction W), and protein displaced by cysteine (fraction I), by GSH (fraction II) and by dithiothreitol (fraction III).

In some preparations of fraction I a white precipitate of insoluble cystine formed in eluted fractions, but this was removed during the standard treatment with dithiothreitol and dialysis. Protein fractions were stored for short periods at 4°C and for long periods at –20°C.

Regeneration of thiopropyl-Sepharose 6B. The recommended method for regeneration of the thiol-binding capacity of thiopropyl-Sepharose 6B is that of Brocklehurst *et al.* (1973). However, this was originally developed for a much less highly substituted covalent chromatography gel, activated thiol-Sepharose 4B (Pharmacia), and is inadequate for the regeneration of thiopropyl-Sepharose 6B.

Satisfactory regeneration was achieved by the method of J. Carlsson [D. Low (Pharmacia), personal communication], which uses a 10-fold higher concentration of oxidant than that of Brocklehurst *et al.* (1973) and is considerably more vigorous. Although this has not been investigated, two reasons can be proposed to explain the more rigorous procedure required in this case. Firstly, a higher concentration of 2,2'-dithiopyridine may be necessary because of the greater extent of substitution in thiopropyl-Sepharose 6B (20 μmol of activated thiol/ml of swollen gel). Secondly, in the aqueous alcoholic reaction mixture the thiol groups on the reduced gel may be present mainly in the less reactive protonated form. A 50 ml portion of gel was fully reduced by incubation with 20 mM-dithiothreitol, pH 8, for 30 min at 30°C, and the gel was then extensively washed at the pump with 1 mM-HCl (5–8 vol.) followed by 1 mM-HCl/1 mM-EDTA (75–100 ml) and 0.2 M-sodium borate buffer (pH 8)/1 mM-EDTA (75–100 ml). After filtration at the pump until moist, the washed gel was transferred to a round-bottomed flask fitted with a reflux condenser and heated to 80°C in a water bath. Then 20 ml of 2,2'-dithiopyridine (160 mM in ethanol or propan-2-ol) was added, and the mixture was heated under reflux at 80°C for 3 h, before the water bath was switched off and the mixture allowed to cool overnight. The gel was then washed at the pump with 5–8 vol. of ethanol or propan-2-ol, 75–100 ml of 0.2 M-sodium borate buffer (pH 8)/1 mM-EDTA and 3–5 vol. of elution buffer (TKM/EDTA/NaCl buffer). Regenerated gel was stored at 4°C and used within 2 days.

Preparation of purified bovine liver protein disulphide-isomerase. The complete procedure for the purification of protein disulphide-isomerase from bovine liver is summarized in Scheme 1.

Enzyme assays

Protein disulphide-isomerase. Protein disulphide-isomerase was routinely assayed by the method of Ibbetson & Freedman (1976), in which re-activation of 'scrambled' RNAase is followed by a dual-

wavelength spectrophotometric assay of ribonuclease activity with highly polymerized yeast RNA as substrate. RNAase activities were obtained from the first 1.5–2 min of the spectrophotometric assay and a plot of RNAase activity versus time of withdrawal of the sample from the isomerase incubation gave a linear time course for up to 15 min. The gradient of this portion of the plot was calculated by linear-regression analysis (correlation coefficient routinely >0.95) and gave the protein disulphide-isomerase activity. Assays were performed in triplicate, and were corrected for the rate of non-enzymic reactivation of 'scrambled' RNAase by dithiothreitol alone. The unit of isomerase activity is that defined by Ibbetson & Freedman (1976).

Standard substrate concentrations in the isomerase assay were $10\mu\text{M}$ -dithiothreitol and $50\mu\text{g}$ of 'scrambled' RNAase/ml. When alternative thiol substrates were used, they were made up as 10 mM stock solutions and were present in the assay mixture at 1 mM. The only exception was reduced bovine serum albumin; this was prepared by incubation of the protein at 30 mg/ml in 10 M-urea at room temperature for 20 h in the presence of 110 mM-dithiothreitol, followed by elution from a column of Sephadex G-25 with 10 mM-acetic acid. Protein and thiol contents were determined by standard methods (Lowry *et al.*, 1951; Ellman, 1959), and the reduced protein preparation was diluted with 10 mM-acetic acid to give a thiol concentration of 1 mM. When used as the thiol component of an assay of protein disulphide-isomerase activity, reduced bovine serum albumin was present at a thiol concentration of 0.1 mM.

The standard assay buffer was 50 mM-Tris/HCl buffer, pH 7.5, containing 25 mM-KCl, 5 mM-MgCl₂ and 1.25 mM-EDTA (TKM/EDTA buffer). For assay of microsomal samples or homogenates, buffers contained 0.25 M-sucrose and were gassed with CO for 15 min before use. In studies on pH-dependence the buffers were 0.2 M-Tris/HCl buffers in the range pH 6.0–8.3 and 50 mM-Tris/HCl buffers in the range pH 8.7–10. For the determination of kinetic parameters the concentration of 'scrambled' RNAase was varied from 5 to $100\mu\text{g}/\text{ml}$ in the presence of $10\mu\text{M}$ -dithiothreitol, and the concentration of dithiothreitol was varied from 1 to $20\mu\text{M}$ in the presence of $50\mu\text{g}$ of 'scrambled' RNAase/ml. Initial-rate data at various substrate concentrations were used to calculate the Michaelis constants and the maximum specific activity. A computer program (Pettit, 1978) gave, by linear-regression analysis, the lines of best fit for the plots of $1/v$ against $1/s$, of s/v against s and of v against v/s . The program also gave estimates of the kinetic parameters by using the direct linear method (Eisenthal & Cornish-Bowden, 1974).

For treatment with iodoacetate, samples of

purified protein disulphide-isomerase ($1.85\mu\text{g}$ of protein) were incubated for 30 min at 30°C in the presence of 5–200 pmol of iodoacetic acid; the total volume was 1 ml and the buffer was TKM/EDTA buffer. In some cases $10\mu\text{M}$ -dithiothreitol was also present. After incubation, samples were frozen overnight, slowly thawed, dithiothreitol was added to $10\mu\text{M}$ and then standard protein disulphide-isomerase assay was initiated by addition of 'scrambled' RNAase. Activities were corrected for the control activities in samples without enzyme, and the results are expressed as percentages of the activity observed in enzyme samples treated in the same way but with the omission of iodoacetate. A similar protocol was followed for studies of the effects of Cd^{2+} ions; the incubation mixtures contained 5–500 pmol of CdCl_2 .

Glutathione-insulin oxidoreductase. This activity was routinely assayed by the method of Ibbetson & Freedman (1976), a linked spectrophotometric assay based on the method of Katzen & Tietze (1966). The standard buffer was TKM/EDTA buffer, but for samples containing microsomal membranes (homogenates, microsomal suspensions etc.) 0.25 M-sucrose was also present and the buffer was gassed periodically with CO to inhibit endogenous NADPH oxidase activity. Assays were performed in duplicate or triplicate; variation between replicates was 5–10% of the mean. All activities were corrected for the non-enzymic control rate (enzyme omitted from both sample and control cuvette), which was also determined in duplicate. The unit of glutathione-insulin oxidoreductase activity is that defined by Ibbetson & Freedman (1976).

Other glutathione-disulphide oxidoreductase activities. Oxidoreduction between GSH and model disulphide compounds was assayed by the method for glutathione-insulin oxidoreductase, with the other disulphides replacing insulin in the assay procedure. All disulphide compounds were used at a disulphide concentration of 0.4 mM. Control incubations were performed for each disulphide and the results were corrected for the non-enzymic rate of oxidoreduction.

Other methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as the standard.

SDS/polyacrylamide-gel electrophoresis was performed in 1.5 mm slab gels by using the buffer system of Laemmli (1970). Uniform 7.5%-acrylamide polyacrylamide gels were used and molecular weights were estimated by using the mobility of chymotrypsinogen A (mol.wt. 23 500), ovalbumin (mol.wt. 43 000) and bovine serum albumin (mol.wt. 68 000) as standards, after staining with Coomassie Brilliant Blue R250.

Results

Resolution of protein disulphide-isomerase and glutathione-insulin transhydrogenase activities

Partial resolution by ion-exchange chromatography. In previous work (Hawkins & Freedman, 1976), protein disulphide-isomerase was purified 140-fold from a bovine liver homogenate by a procedure involving acetone extraction of a microsomal fraction, buffer extraction of the resultant acetone-dried powder, $(\text{NH}_4)_2\text{SO}_4$ precipitation and three stages of ion-exchange chromatography. The resultant material also had some glutathione-insulin transhydrogenase activity, and this and the isomerase activity were not resolved at any stage. Nevertheless the activities did not co-purify quantitatively, implying that distinct enzymes were involved, which would be resolved by appropriate techniques.

We have re-examined this, taking as starting material preparations purified from bovine liver up to the elution from DEAE-Sephadex A-50 at pH 7.8, in the procedure of Hawkins & Freedman (1976). Such preparations have a protein disulphide-isomerase activity of 50–70 units/g and glutathione-insulin transhydrogenase activity of 6–9 units/g; they show eight to ten bands on SDS/polyacrylamide-gel electrophoresis. This material was applied to columns of DEAE-Sephadex A-50 at pH 6.3 and eluted with increasing concentrations of NaCl by using either gradients or stepwise elution procedures. In the gradient runs the peaks of glutathione-insulin transhydrogenase activity and protein disulphide-isomerase activity were not coincident, the isomerase peak occurring at a higher ionic strength. This was confirmed in a series of stepwise elutions, involving different numbers of steps and different salt concentrations. In every case glutathione-insulin transhydrogenase activity was preferentially eluted at salt concentrations in the range 0.2–0.26 M, whereas protein disulphide-isomerase was concentrated in fractions eluted at 0.35–0.50 M. In none of these experiments was complete resolution achieved: fractions high in isomerase activity also had some glutathione-insulin transhydrogenase

activity, and vice versa. On examination by SDS/polyacrylamide-gel electrophoresis, fractions high in protein disulphide-isomerase activity were heterogeneous, but the major band in every case was at a position corresponding to a mol.wt. of 57 000–58 000. Fractions rich in glutathione-insulin transhydrogenase activity had bands corresponding to mol.wts. of 69 000 and 38 000.

Although these experiments with ion-exchange chromatography did not succeed in fully resolving the enzyme activities, they showed clearly that they were physically separable. These experiments stimulated the development of an alternative separative technique.

Complete resolution by covalent chromatography. The partially purified material described in the preceding section (eluted from DEAE-Sephadex A-50 at pH 7.8) was gently reduced and freed from reducing agent by gel filtration (see the Experimental section). It was then applied to activated thiopropyl-Sepharose 6B, an agarose gel containing the functional group $-\text{O}-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2-\text{S}-$ (S-Ar), where (S-Ar) is the 2-thiopropylidyl leaving group. Proteins containing reactive thiols react with this functional group, displacing 2-thiopyridone and becoming bound to the gel as mixed disulphides. Unbound proteins were displaced from the column by extensive buffer washes, and the bound material was then fractionated by a new sequential elution procedure involving three steps of increasing reducing power. The procedure resulted in collection of four fractions of protein, namely material not bound to the column (fraction W), protein displaced by 20 mM-L-cysteine (fraction I), protein displaced by 50 mM-GSH (fraction II) and protein displaced by 20 mM-dithiothreitol (fraction III). Protein recovered in these four fractions was on average 73% of that applied (range 58–91% in six experiments). Approximately half the recovered protein was in fraction W and the remainder was equally distributed between fractions I, II and III.

The fractions were assayed for protein disulphide-isomerase and glutathione-insulin transhydrogenase activities. The results (Table 1) show that the procedure reproducibly resolves the two

Table 1. *Resolution of protein disulphide-isomerase and glutathione-insulin transhydrogenase by covalent chromatography*

Results are shown from three small-scale covalent chromatography runs with the same starting material (DEAE-Sephadex pH 7.8 eluate). Experimental details are given in the text. N.D., Not detectable.

Fraction	Protein disulphide-isomerase (units/g)			Glutathione-insulin transhydrogenase (units/g)		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Fraction W	35.4	32.5	46.6	8.0	8.1	1.8
Fraction I	57.1	19.5	14.8	N.D.	N.D.	N.D.
Fraction II	N.D.	N.D.	N.D.	1.1	N.D.	N.D.
Fraction III	N.D.	N.D.	N.D.	7.9	1.4	0.5

enzymic activities. Both activities are found in the unbound material (fraction W), probably as a result of overloading or incomplete reaction. In a large-scale preparative experiment (see below) involving a different loading protocol, much lower enzyme activities were found in this fraction. Fraction I showed protein disulphide-isomerase activity and no detectable glutathione-insulin transhydrogenase activity. Fraction III, by contrast, showed glutathione-insulin transhydrogenase activity but no protein disulphide-isomerase activity. In one run fraction II showed just detectable glutathione-insulin transhydrogenase activity, but in general this fraction had neither enzymic activity. All fractions were subjected to the same work-up before assay (treatment with dithiothreitol to reduce any mixed disulphides between protein and eluting reagent, followed by extensive dialysis), so that the differences in activity between the fractions can only reflect the presence of distinct proteins differing in enzymic activity. This is confirmed by SDS/polyacrylamide-gel electrophoresis; fraction I, which has protein disulphide-isomerase activity, showed two major bands at mol.wts. 57 000–59 000 and 70 000–72 000, whereas fraction III, which has glutathione-insulin transhydrogenase activity, showed one main band at mol.wts. 36 000–38 000, sometimes also with a band at mol.wts. 70 000–72 000.

Although the activities were clearly resolved by this procedure, they were not extensively purified, in terms of specific activities. This probably reflects a significant degree of inactivation after the rather complex and time-consuming sequence of processes involved, namely reduction, loading, stepwise elutions and work-up (see the Experimental section for details). Eluted fractions were generally assayed 4–7 days after the beginning of the experiment. Nevertheless the successful resolution prompted an attempt at large-scale resolution by a similar procedure.

Purification of protein disulphide-isomerase by preparative-scale covalent chromatography and ion-exchange chromatography. In order to purify

protein disulphide-isomerase on a preparative scale, 125 mg of freeze-dried protein corresponding to the starting material described in the two preceding sections (DEAE-Sephadex pH 7.8 eluates) was dissolved in 10 ml of 20 mM-phosphate buffer, pH 6.3, reduced with 0.1 mM-dithiothreitol, eluted from Sephadex G-25 and then coupled to 50 ml of activated thiopropyl-Sepharose 6B by overnight incubation at 25°C. The loaded gel was packed into a column (2.5 cm × 15 cm) and eluted by using the sequential procedure described above. Fractions W, I, II and III were collected and worked up as before. The total recovery of protein was 69%; of the recovered material 20% was in fraction I, 4% in fraction II and 9% in fraction III.

The worked-up fractions were assayed for protein disulphide-isomerase and glutathione-insulin transhydrogenase activities (Table 2). As before, the activities were resolved, the former appearing in fraction I and the latter in fraction III, but the results were not identical with those in the analytical-scale covalent-chromatography runs. Fraction W (unbound protein) only showed a low protein disulphide-isomerase activity, indicating much more successful coupling of the enzymes to the column in this procedure. Fraction I showed a high protein disulphide-isomerase activity, as before (cf. Table 1), but also showed some glutathione-insulin transhydrogenase activity. Fractions II and III were comparable with previous elutions (Table 1), the former showing little detectable enzyme activity and the latter showing glutathione-insulin transhydrogenase activity only. The rather poorer resolution of this larger-scale procedure was compensated by the much greater recovery of protein disulphide-isomerase activity. Fraction I had a considerably higher specific activity than in the smaller-scale experiments and was purified significantly (Table 2). As before, in SDS/polyacrylamide-gel electrophoresis fraction I showed two major bands at mol.wts. about 58 000 and 70 000, but several minor bands were also present.

Since this large-scale covalent-chromatography

Table 2. *Purification of protein disulphide-isomerase by covalent chromatography*
Experimental details are given in the text. N.D., Not detectable.

Fraction	Protein disulphide-isomerase			Glutathione-insulin transhydrogenase		
	Specific activity (units/g)	Purification factor	Yield (%)	Specific activity (units/g)	Purification factor	Yield (%)
DEAE-Sephadex pH 7.8 eluate	32.1	(1)	(100)	1.53	(1)	(100)
Fraction W	8.1	0.25	7.6	N.D.	—	0
Fraction I	215.6	6.71	92.7	2.72	1.78	10.1
Fraction II	1.4	0.05	0.1	N.D.	—	0
Fraction III	N.D.	—	0	1.61	1.05	14.4

experiment had not fully resolved the two enzymic activities, fraction I was concentrated by ultrafiltration and subjected to ion-exchange chromatography on DEAE-Sephadex A-50 at pH 6.3 by using a protocol similar to those described above under 'Resolution of protein disulphide-isomerase and glutathione-insulin transhydrogenase activities: Partial resolution by ion-exchange chromatography' which had partially separated the two activities when applied to the DEAE-Sephadex pH 7.8 eluate. Fig. 1 shows the elution profile and indicates the fractions D1, D2 and D3,

Fig. 1. Final purification of protein disulphide-isomerase by ion-exchange chromatography

A sample of protein disulphide-isomerase purified by covalent chromatography (15 mg, fraction I) was loaded on to a column of DEAE-Sephadex A-50 (2.5 cm $\times$ 5 cm) at pH 6.3, and was then eluted with a series of solutions of phosphate buffer (20 mM, pH 6.3) containing increasing concentrations of NaCl. Fractions of volume 2 ml were collected and monitored for absorbance at 280 nm (—) and NaCl concentration (----).

which were retained for analysis. These fractions were assayed for protein and enzyme activities (Table 3). As described above, the activities are resolvable by ion-exchange chromatography, with protein disulphide-isomerase being eluted at a higher ionic strength than is glutathione-insulin transhydrogenase. Thus fraction D3 has a very high specific activity of protein disulphide-isomerase and only barely detectable glutathione-insulin transhydrogenase activity. Relative to a crude bovine liver homogenate, this material is 560-fold purified in protein disulphide-isomerase activity but is only 1.1-fold purified in glutathione-insulin transhydrogenase activity. On SDS/polyacrylamide-gel electrophoresis, this material showed a single major band at mol.wt. 57 000, but it was not completely homogeneous and four additional bands were detectable at high loadings.

Preliminary characterization of purified protein disulphide-isomerase

The isolation of protein disulphide-isomerase by the combination of covalent chromatography and ion-exchange chromatography results in a preparation of the enzyme that is almost homogeneous and has a much higher specific activity than the previously available preparations (Hawkins & Freedman, 1976). The complete purification schedule is briefly summarized in Scheme 1. Since only a small amount of this highly purified material was available, only a preliminary characterization can be given here. In this we have concentrated on the catalytic properties of the enzyme.

Kinetic parameters. The standard assay for protein disulphide-isomerase, used above and in the bulk of our previous work (Ibbetson & Freedman, 1976; Hawkins & Freedman, 1976; Freedman *et al.*, 1978; Harwood & Freedman, 1978), employs 50 μ g of 'scrambled' RNAase/ml and 10 μ M-dithiothreitol, and is performed at pH 7.5 and 30°C. These

Table 3. Further purification of protein disulphide-isomerase by ion-exchange chromatography after covalent chromatography

Experimental details are given in the text. N.D., Not detectable.

Fraction	Protein disulphide-isomerase			Glutathione-insulin transhydrogenase		
	Specific activity (units/g)	Purification factor	Yield* (%)	Specific activity (units/g)	Purification factor	Yield* (%)
DEAE-Sephadex pH 7.8 eluate	32.1	(1)	—	1.53	(1)	—
Fraction I from covalent chromatography	215.6	6.71	(100)	2.72	1.78	(100)
Fraction D1	0.75	0.02	0.01	N.D.	—	0
Fraction D2	73.4	2.29	5.5	5.85	3.82	34.4
Fraction D3	555.4	17.3	51.3	0.52	0.34	3.7

* Expressed as percentage of total activity applied to the DEAE-Sephadex column (pH 6.3).

Scheme 1. Summary of procedure for purification of bovine liver protein disulphide-isomerase

conditions were derived from preliminary kinetic work on the enzyme in a number of crude sources and various partially purified materials. The availability of pure enzyme made it imperative to carry out a more detailed kinetic study. Experiments in which each of the substrate concentrations were separately varied are summarized in Fig. 2. Since in both cases the plots correspond fairly well to rectangular hyperbolae, the data were analysed by the conventional linear plots and by the direct linear plot (Eisenthal & Cornish-Bowden, 1974), which is generally regarded as the most reliable method for the determination of K_m and V_{max} . (Henderson, 1978). For both substrates the parameters derived from the Lineweaver–Burk plots do not agree closely with those derived from the other methods, but the parameters derived from the plots of s/v against s and of v against v/s agree closely with each other and with those from the direct linear plot. Thus for ‘scrambled’ RNAase as variable substrate these

last three methods give the following results: for K_m 26.5, 21.2 and $20.4 \mu\text{g/ml}$ respectively, and for the maximum specific activity 780, 722 and 759 units/g respectively. For dithiothreitol as variable substrate these three methods give the following results: for K_m 4.90, 5.70 and $5.66 \mu\text{M}$ respectively, and for maximum specific activity 711, 737 and 713 units/g respectively. The continuous lines in Fig. 2 are those corresponding to the mean values of these estimates, namely $22.7 \mu\text{g/ml}$ and 754 units/g for Fig. 2(a) and $5.42 \mu\text{M}$ and 721 units/g for Fig. 2(b). The close correspondence between the values obtained by the different methods of data analysis indicates that the data with respect to both substrates are adequately described by the Michaelis–Menten equation. The substrate concentrations employed in the standard assay conditions are close to twice the derived values of K_m , indicating that the standard assay conditions are satisfactory for general work.

pH optimum. The purified material was assayed in

Fig. 2. Kinetic characterization of purified protein disulphide-isomerase

The basic assay method is described in the Experimental section. (a) Variation of activity with dithiothreitol concentration; the concentration of 'scrambled' RNAase was 50 $\mu\text{g/ml}$. (b) Variation of activity with 'scrambled' RNAase concentration; the concentration of dithiothreitol was 10 μM . A 1.85 μg portion of purified protein disulphide-isomerase was present in each assay. The continuous lines are those calculated by using the values for kinetic constants given in the text.

the standard conditions except that the standard assay buffer was replaced by 0.2 M-Tris/HCl buffers covering the pH range 6.0–8.3 and 50 mM-Tris/HCl buffers covering the pH range 8.7–10.0. Appropriate control assays were performed for each buffer. Enzyme activity was detected in the range pH 6.4–8.3 and the maximum activity was observed at pH 7.5. Activities greater than 50% of the maximum were observed in the range pH 7.2–8.0, emphasizing the narrow pH range of this enzyme.

Thiol specificity. The enzyme was assayed with various monothiols replacing dithiothreitol. GSH, cysteamine, 2-mercaptoethanol, thioglycolic acid and L-cysteine are all effective, but significantly higher concentrations of these monothiols are required, compared with dithiothreitol. The monothiols at 1 mM have activities in the range 20–80% that found with 10 μM -dithiothreitol.

Inhibition by iodoacetate. Considerations of plausible mechanisms for an enzyme catalysing thiol–disulphide interchange suggest that protein disulphide-isomerase may have an active-site thiol group, and there is some evidence for an essential thiol group (DeLorenzo *et al.*, 1966a,b; Fuchs *et al.*, 1967). The experiments involving covalent chromatography described above imply the presence of an accessible thiol group. To test whether the enzyme purified now contains an essential thiol group,

purified protein disulphide-isomerase (1.85 μg , 32.5 pmol) was incubated with iodoacetate in the range 5–200 pmol at pH 7.5 at 30°C for 30 min before assay in conventional conditions. The enzyme was inactivated by exposure to iodoacetate, and complete inactivation was seen at iodoacetate amounts above 80–90 pmol (i.e. 2–3-fold molar excess over enzyme). At smaller iodoacetate amounts the behaviour was not simple; very small amounts of iodoacetate (5–10 pmol) produced 50% inactivation, and amounts of iodoacetate up to 50 pmol produced no additional inactivation. Above 50 pmol of reagent, inactivation increased continuously to be complete at 80–90 pmol. The reasons for this biphasic behaviour require further investigation, but the significant finding is that the enzyme is inactivated by close to stoichiometric quantities of iodoacetate in conditions where thiol groups alone are likely to be modified. To test whether any essential thiol groups might be masked in the free enzyme and require the presence of dithiothreitol to become available for modification, the above experiment was also performed in the presence of 10 μM -dithiothreitol. As before, there was biphasic inactivation as a function of iodoacetate concentration, and the enzyme was fully inactivated after treatment with 80–90 pmol of iodoacetate. These preliminary chemical-modification data imply that.

both in the absence and presence of reducing agents, the enzyme molecule contains two or three thiol groups that are available for modification by iodoacetate and essential for activity.

Inhibition by Cd^{2+} . In very early work on protein disulphide-isomerase activity in crude sources (Ramakrishna Kurup *et al.*, 1966) it was suggested that the enzyme might contain an essential vicinal dithiol group. This is consistent with the observations on inactivation by iodoacetate (above) and also with work on the details of the catalytic action of purified protein disulphide-isomerase on bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1980). Several enzymes contain such an essential dithiol group, including another thiol-disulphide-interchange enzyme (Carmichael *et al.*, 1979), and inhibition by low concentrations of Cd^{2+} is regarded as diagnostic of such enzymes (Gaber & Fluharty, 1968).

Treatment of purified protein disulphide-isomerase with low concentrations of this ion produced significant inhibition (Fig. 3). Very small quantities of Cd^{2+} (5 pmol, concentration in the incubation

Fig. 3. Inhibition of purified protein disulphide-isomerase by Cd^{2+} ions

Samples of protein disulphide-isomerase (1.85 μg , 32.5 pmol) in TKM/EDTA buffer were incubated in the presence of various amounts of Cd^{2+} (samples from a stock solution of 1 μM - CdCl_2) at 30°C for 30 min, frozen overnight, thawed and then assayed by conventional procedure. □, No additions during incubation with Cd^{2+} ; ■, 10 μM -dithiothreitol present during incubation with Cd^{2+} .

medium 5 nM) reproducibly caused slight activation of the enzyme, but all other concentrations tested were inhibitory. Complete inhibition was not achieved, and 15–30% activity remained at all cases where Cd^{2+} was in molar excess over the enzyme. The residual activity may be due to the presence of 1.25 mM-EDTA in the incubation buffer, since it has been reported that EDTA partially reverses the inhibitory effect of Cd^{2+} on dithiol enzymes (Gaber & Fluharty, 1968; Carlson *et al.*, 1978). The observation of extensive inhibition by low concentrations of Cd^{2+} is strong evidence for the presence of an essential dithiol group in the enzyme.

Thiol and disulphide specificities of bovine liver thiol-protein disulphide oxidoreductase and protein disulphide-isomerase

The results given above under 'Resolution of protein disulphide-isomerase and glutathione-insulin transhydrogenase activities' establish conclusively that, in bovine liver, there is not a single enzyme responsible for both protein disulphide-isomerase and glutathione-insulin transhydrogenase activities. However, these results do not indicate how many such enzyme species contribute to the observed activities in bovine liver homogenates. The data above cannot give any information on this question, and only limited information is available from the literature. It is possible that several enzymes with rather different specificities are present in tissues (Freedman, 1979). We have tested this by assaying a range of thiol-disulphide interchange activities in a bovine liver homogenate and in parallel at several stages of purification from that source.

Protein disulphide-isomerase activities were measured with 'scrambled' RNAase as disulphide substrate and a variety of thiol substrates; these included several monothiols differing in molecular size and acid/base properties, and a reduced protein containing multiple thiol groups, as well as the standard thiol substrate, dithiothreitol. Thiol concentrations were selected to give roughly similar enzymic rates. The glutathione-insulin transhydrogenase assay was performed with various disulphides replacing insulin, so that several glutathione-disulphide transhydrogenase activities could be compared. The disulphides included low-molecular-weight species, proteins containing disulphides stable to disulphide rearrangement (ribonuclease, serum albumin) and proteins with metastable disulphide bonds (insulin, 'scrambled' RNAase). In all, 12 thiol-disulphide interchange activities were monitored through several stages of the conventional purification of protein disulphide-isomerase from bovine liver.

Results at three stages of purification are shown in Figs. 4 and 5. With a single exception, the protein disulphide-isomerase activities with the various

Fig. 4. Activity of protein disulphide-isomerase with various thiols

Isomerase activity was determined in (a) bovine liver homogenate, (b) microsomal fraction and (c) a purified enzyme preparation obtained by chromatography of 'DEAE pH 7.8 eluate' on DEAE-Sephadex A-50, in 20 mM-phosphate buffer, pH 6.3, with a linear NaCl gradient. Various thiols were used as co-substrate, namely reduced bovine serum albumin (concn. of SH group 0.1 mM) (A), cysteine (1 mM) (B), dithiothreitol (10 μ M, concn. of SH group 20 μ M) (C), β -mercaptoethanol (1 mM) (D), thioglycolic acid (1 mM) (E) and GSH (1 mM) (F).

Fig. 5. Glutathione-disulphide oxidoreductase activity assayed with various disulphides

Glutathione-disulphide oxidoreductase activities were assayed by the linked spectrophotometric procedure (see the Experimental section) in the same samples (a), (b) and (c) referred to in the legend to Fig. 4. All disulphide compounds were present in assay mixtures at a disulphide concentration of 0.4 mM. The disulphides used were cystine (A), 'scrambled' (randomly reoxidized) RNAase (B), *trans*-4,5-dihydroxy-1,2-dithiane (oxidized dithiothreitol) (C), bovine serum albumin (D), insulin (E) and native RNAase (F).

thiols show close quantitative co-purification. Thus, with the thiol concentrations selected, the order of preference for thiol substrates is reduced bovine serum albumin > dithiothreitol > 2-mercaptoethanol > thioglycolic acid > GSH for the crude homogenate, for microsomal fraction and for the final material, which is approximately 50-fold purified in all these activities. This strongly implies that a single major enzymic species is responsible for most of the protein disulphide-isomerase activity observed in bovine liver. Cysteine is an effective thiol substrate for protein disulphide-isomerase in the homogenate, but not in microsomal fraction or at any later stage of purification. This implies that an enzyme species with a strong preference for cysteine is present in the homogenate but is removed during the differential centrifugation procedures.

In contrast with the protein disulphide-isomerase activities, the glutathione-disulphide oxidoreductase activities were not significantly purified by this procedure and did not show a consistent pattern of purification. No two activities showed close quantitative co-purification. This is illustrated in Fig. 5, where it is clear that the order of disulphide substrate preference is not maintained through the purification. No clear conclusions can be drawn from these results, except that it is likely that bovine liver contains several enzymes capable of catalysing glutathione-disulphide oxidoreduction, each with rather different, though probably overlapping, substrate preferences.

Discussion

Covalent chromatography

Thiol-disulphide interchange reactions between molecules in solution and groups immobilized on agarose gels form the basis of the technique known as covalent chromatography (Brocklehurst *et al.*, 1974). Two preparations for covalent chromatography are commercially available, 'activated thiol-Sepharose 4B' and 'thiopropyl-Sepharose 6B'; the latter preparation, which has a much higher available thiol group content, was used in the present work. The technique has been extensively used in the purification of thiol-containing proteins, to separate proteins with reactive thiol groups from those lacking this group (for review see Brocklehurst, 1979); it has also been used to isolate thiol-containing peptides from digests of several proteins (Egorov *et al.*, 1975; Svenson *et al.*, 1977). It has been recognized for some time that the technique should potentially be able to resolve distinct thiol-containing proteins (see, e.g., Brocklehurst *et al.*, 1974; Laurell *et al.*, 1977), but this application has not been extensively explored.

Early work on the chemical modification of protein disulphide-isomerase (DeLorenzo *et al.*,

1966a,b) and of glutathione-insulin transhydrogenase (Varandani & Plumley, 1968) indicated that reactive thiol groups were essential for both activities. Indeed these findings were quoted as evidence that a single enzyme was responsible for both activities (Varandani, 1974). The existence of such reactive thiol groups implied that the protein(s) responsible for these activities should be able to be coupled to covalent chromatography gels. If distinct proteins were involved, the thiols involved in linkage to the matrix would differ to some extent in chemical properties owing to their different protein micro-environments; the resultant protein-matrix mixed disulphides might then show different reactivities to added thiols and the proteins would require rather different reducing conditions for release from the gel.

The strong circumstantial evidence that these two activities were not alternative expressions of a single enzyme (Ibbetson & Freedman, 1976; Hawkins & Freedman, 1976; Freedman *et al.*, 1978; Hillson, 1979), and the difficulties in obtaining complete resolution of these activities by conventional methods, led us to attempt resolution by covalent chromatography. A stepwise elution protocol was selected as likely to give sharper resolution and more concentrated eluted fractions. Rather than use several concentrations of a single eluent it was decided to use three different eluents, two physiological monothiols followed by a powerfully reducing dithiol. The two monothiols were chosen so that the fractionation would exploit not only differences in chemical reactivity between the bound proteins, but also any differences in biological specificity. Thus GSH, a substrate for one of the activities, was chosen so that the technique might be considered a combination of covalent chromatography and affinity chromatography. Despite the tenuous reasoning, the elution protocol thus derived was successful at resolving these two enzyme activities in a partially purified preparation from bovine liver (Tables 1 and 2). The protocol can also be used successfully to resolve these activities in a much cruder preparation from bovine liver (see Hillson & Freedman, 1980), emphasizing that the technique is comparable with affinity chromatography in its possibilities.

Protein disulphide-isomerase is very easily displaced from its covalent interaction with the gel and is reproducibly obtained in fraction I (displaced by 20 mM-L-cysteine). This lability of the isomerase-gel mixed disulphide is not surprising if the thiol group involved in the linkage is one that is also involved in the catalytic activity of the enzyme. The mechanism of the enzyme remains to be determined (see the Discussion section in Creighton *et al.*, 1980), but it is likely that the enzyme forms mixed disulphides with its protein substrates, facilitating the rearrangement of their disulphide bonds. For

effective catalysis, it would be important that the active-site thiol group of the enzyme was a good leaving group as well as a good attacking nucleophile. Glutathione-insulin transhydrogenase was not displaced by its substrate GSH (at 50 mM) but required the stronger reducing conditions provided by 20 mM-dithiothreitol, indicating that this enzyme forms quite a stable mixed disulphide with the thiopropyl group of the gel.

Resolution of enzymes by covalent chromatography obviously merits further systematic investigation. Our priority in the present work was the purification of protein disulphide-isomerase, and we achieved this with some serendipity and without extensive exploration of different elution protocols. Nevertheless it seems likely that, with further basic work, this technique could be developed so that it can be applied rationally in the resolution and purification of proteins containing reactive thiol groups.

Protein disulphide-isomerase and glutathione-insulin transhydrogenase are distinct enzymes

The action of protein disulphide-isomerase was first described in the early 1960s (Goldberger *et al.*, 1963; Givol *et al.*, 1964; Venetianer & Straub, 1963, 1964), and some structural data were later published (DeLorenzo *et al.*, 1966*a,b*; Fuchs *et al.*, 1967). Glutathione-insulin transhydrogenase was characterized at about the same time (Tomizawa & Halsey, 1959; Tomizawa, 1962; Katzen & Tietze, 1966), and similarities were noted between the properties of this enzyme and those of protein disulphide-isomerase. Since the reactions catalysed by both enzymes involve interchange between thiols and protein disulphides, it was natural to speculate that a single enzyme might be capable of catalysing both reactions. Purified preparations of the transhydrogenase were found capable of reactivating 'scrambled' RNAase (Katzen & Tietze, 1966; Varandani, 1973; Ansoorge *et al.*, 1973*a*), and this led to the suggestion that there was only a single enzyme that was responsible for both activities. Apart from this overlap of catalytic activities, however, there has been little direct comparison of the two activities. Data on one activity have been compared with published data on the other, often involving a different tissue or species. The data quoted in support of the 'single-enzyme' hypothesis (summarized in Varandani, 1974, 1978) are essentially (i) that the activities involve similar reactions, (ii) that both activities are found in the microsomal fraction (where this has been determined), (iii) that both activities involve an essential thiol group and (iv) that there are similarities in amino acid composition between protein disulphide-isomerase purified from bovine liver in one laboratory and glutathione-insulin transhydrogenase purified in two

other laboratories from rat liver, bovine liver and bovine pancreas. Other structural data on the purified enzymes are rather equivocal (see, e.g., Morin *et al.*, 1978).

It is now clear that there are a number of cellular processes involving thiol-protein disulphide interchange reactions, including enzyme regulation (Rodriguez & Pitot, 1976; Holmgren *et al.*, 1977), maintenance of the cellular proportions of GSH, GSSG and GSS-protein (Isaacs & Binkley, 1977; Axelsson *et al.*, 1978) as well as formation of disulphide bonds in assembly of multi-chain proteins (Della Corte & Parkhouse, 1973; Richardson & Feinstein, 1978) and degradation of polypeptide hormones (Varandani & Nafz, 1976). It therefore seems likely that there may be several enzymes catalysing such reactions (Freedman, 1979); if so, it would not be surprising if such enzymes had certain features in common, such as an ability to act on several thiol and disulphide substrates and the possession of a reactive thiol group essential for catalytic activity.

We can therefore distinguish two hypotheses: (a) that a single enzyme is responsible for the two activities commonly known as protein disulphide-isomerase and glutathione-insulin transhydrogenase, or (b) that there exist two or more distinct enzymes catalysing thiol-protein disulphide interchange reactions, with distinct specificities and physiological roles. Rigorous testing of these hypotheses requires quantitative data obtained from parallel study of the two activities. In particular, the hypotheses differ in a number of predictions. The former suggests (1) that the two activities will show identical subcellular and tissue distributions, (2) that the two activities will show the same responses to heat and other denaturing treatments, (3) that the two activities will co-purify quantitatively when assayed in parallel during a purification from a crude source, and (4) that the two activities will not be resolvable by any method. The hypothesis (b) makes exactly converse predictions.

In all cases where these criteria have been employed in parallel studies on the two activities, the data support hypothesis (b) and are inconsistent with hypothesis (a). (1) Data have been published on the relative activities of protein disulphide-isomerase in bovine tissue microsomal fractions (DeLorenzo & Molea, 1967) and on the relative activities of glutathione-insulin transhydrogenase in supernatant fractions from rat tissues (Chandler & Varandani, 1972). Because of the differences in species and subcellular fractions employed, no firm conclusions can be drawn, but the order of activities is quite different for the two activities (see the Discussion section in Varandani, 1974). More recently the two activities have been studied in parallel in subcellular fractions from several sheep

tissues (Hillson, 1979). Again the tissue distributions were found to be quite different. (2) Bovine and rat liver microsomal fractions show both activities. In these sources and in solubilized partially purified preparations from them, the two activities show quite different responses to heat treatment and to treatments with EDTA, detergents and organic solvents (Ibbetson & Freedman, 1976; Hawkins & Freedman, 1976; Freedman *et al.*, 1978). Such findings (particularly those for the soluble preparations, where no differences in permeability can be invoked) cannot be reconciled with hypothesis (a) even if multiple substrate and effector sites are postulated *ad hoc* (Varandani, 1978). (3) The two activities were monitored through a conventional purification of protein disulphide-isomerase from bovine liver (Hawkins & Freedman, 1976). The activities did not co-purify quantitatively at any stage, and the final product was 143-fold purified in protein disulphide-isomerase activity and 10-fold purified in glutathione-insulin transhydrogenase activity. (4) Clearly the strongest criterion of identity is whether the activities are resolvable or not. In fact it has been claimed (Varandani, 1978) that the failure to resolve these activities in the work cited in (3) was the strongest evidence in favour of the 'single-enzyme' hypothesis. In the present paper we have now shown that the two activities are resolvable by covalent chromatography and, to some extent, by ion-exchange chromatography. By a combination of these techniques preparations can be obtained with high specific activities of protein disulphide-isomerase and no, or barely detectable, glutathione-insulin transhydrogenase activity (Tables 1 and 2). Fractions are likewise obtained with transhydrogenase activity and no detectable protein disulphide-isomerase activity. In the following paper (Hillson & Freedman, 1980) we show that this resolution can also be obtained in a cruder preparation from bovine liver obtained by a quite different method. The fractions showing these clear differences in activity show corresponding differences in polypeptide composition as determined by SDS/polyacrylamide-gel electrophoresis.

The overwhelming weight of evidence is therefore in favour of hypothesis (b).

It is important to stress that this conclusion is not inconsistent with the finding that pure preparations of glutathione-insulin transhydrogenase can catalyse reactions characteristic of protein disulphide-isomerase. As with other groups of enzymes, enzymes catalysing exchange reactions between thiols and protein disulphides may show broad and overlapping specificities. The important point is that the activities of a pure enzyme in various assays must be compared with the activities of a crude source. Our dilute preparations of highly purified protein disulphide-isomerase show no detectable

activity in transhydrogenase between GSH and insulin (see Table 1), but concentrated active preparations show just detectable activity. Thus the purified material is 560-fold purified in isomerase activity and 1.1-fold purified in transhydrogenase activity; this low remaining activity may be the result of trace contamination, but it may also represent the intrinsic activity of protein disulphide-isomerase in catalysing transhydrogenation between GSH and insulin. What is clear is that this activity cannot account for all the glutathione-insulin transhydrogenase activity of a bovine liver homogenate. Likewise it is possible that highly purified preparations of glutathione-insulin transhydrogenase may show some activity in the isomerization of disulphide bonds within proteins. However, no quantitative data have been presented on the 'isomerase' activity of purified preparations of glutathione-insulin transhydrogenase, and, until the relative purification factors for the two activities of such preparations are available, no conclusions can be drawn on the significance of their reported isomerase activities.

Possible existence of multiple thiol-disulphide oxidoreductases

The evidence cited above, although ruling out the 'single-enzyme' hypothesis, does not establish that there are simply two enzymes; several enzymic species with different specificities might contribute to each of the observed activities. The findings involving assays with several thiol and disulphide substrates are relevant to this question. In its action on 'scrambled' RNAase, protein disulphide-isomerase requires a reduced thiol as co-substrate (Givol *et al.*, 1964; Drazic & Cottrell, 1977). If several distinct enzymes contributed to isomerase activity, they might be expected to have different preferences for thiol co-substrate. If a single enzyme is responsible for all the measured activities, the activities will co-purify quantitatively; if several species are involved, even if they are not resolved they will show different quantitative extents of purification. This approach has been used previously to establish the number of enzymic species responsible for glutathione *S*-transferase activities (Jakoby & Keen, 1977), for UDP-glucuronyltransferase activities (Burchell, 1978; Wishart, 1978a,b) and for epoxide hydratase activities (Oesch *et al.*, 1971). In fact we find, with five thiol compounds, that the substrate preference is approximately constant through purification (Fig. 4); the discrepant result with cysteine may indicate a contribution from cysteine-dependent cytoplasmic enzymes to the isomerase activity of whole homogenates. The low-molecular-weight cytoplasmic 'thioltransferase' purified by Mannervik and co-

workers (Axelsson *et al.*, 1978) and the cysteine-dependent transhydrogenase (States & Segal, 1969) are possible candidates. The results are not conclusive, but suggest that a single enzyme species, whose purification is described above, is responsible for most of the protein disulphide-isomerase activity of bovine liver.

By the same argument, if a single enzyme species were responsible for all the observed glutathione-insulin transhydrogenase activity, this enzyme would be expected to have a defined specificity for alternative disulphide substrates and the preference for such substrates would not change through purification. The results obtained with six different disulphide substrates are not consistent with this view. Quite different degrees of purification are obtained for the six substrates at each stage (Fig. 5), and no obvious pattern emerges. These data suggest that several thiol-disulphide oxidoreductases may be present in bovine liver, and that the measured glutathione-insulin transhydrogenase activity in crude preparations may involve contributions from several such species.

It is important to note that the most purified preparation in this experiment had not been subjected to covalent chromatography. Nevertheless, it was far more purified in protein disulphide-isomerase activities (approx. 50-fold with five different thiol substrates) than in any of the glutathione-disulphide oxidoreductase activities, which were all less than 4-fold purified. This is seen most strikingly in assays of the different reactions with the same pair of substrates, GSH and 'scrambled' RNAase. The purified preparation is effective in catalysing the reactivation of 'scrambled' RNAase with GSH as co-substrate, but there is little net oxidoreduction, as monitored by the formation of GSSG. This emphasizes that the purified isomerase has a preferential catalytic action on intramolecular thiol-disulphide interchanges within proteins and is a poor catalyst of simple oxidoreductions involving monothiols and protein disulphides. Thus in the nomenclature of Freedman (1979) it is a good catalyst of reactions of type 4 but a poor catalyst of reactions of type 1. The same conclusion has been reached from detailed studies on the catalysis by the purified enzyme of unfolding and refolding of bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1980).

Properties of the purified protein disulphide-isomerase

Because of limitations on the amount of material available, few structural properties of the enzyme have been determined. All the results obtained by SDS/polyacrylamide-gel electrophoresis of purified and partially purified preparations indicate that protein disulphide-isomerase contains a single type of polypeptide chain of mol.wt. $57\,000 \pm 1000$; the

same conclusion is drawn in the following paper (Hillson & Freedman, 1980) from a preparation purified by a different method.

The enzyme requires the presence of a thiol compound and has a broad specificity for thiol compounds, although dithiols and multithiols appear to be effective at much lower concentrations. The K_m for dithiothreitol is approx. $5\,\mu\text{M}$, and the K_m in terms of disulphide bond concentration, for the standard substrate 'scrambled' RNAase, is $7\,\mu\text{M}$. The pH range is narrow. Chemical-modification data point to the presence of an essential thiol or dithiol group. Suggestions for the role of these groups are presented elsewhere (Creighton *et al.*, 1980).

'Scrambled' RNAase is a heterogeneous substrate, and in the conventional assay the appearance of native ribonuclease is monitored, so that the numerous complex disulphide interchange steps in this process are masked. The mechanism of action of the enzyme can better be studied with a simpler, better-characterized, substrate. Studies of the pure enzyme's action on the folding and unfolding of bovine pancreatic trypsin inhibitor (Creighton *et al.*, 1980) have shown that the steps catalysed by the enzyme are those in which a thiol group on the substrate protein attacks a disulphide bond in the substrate protein to produce an intramolecular thiol-disulphide exchange. These are in general the rate-determining steps in protein disulphide formation *in vitro*; the finding supports the view that the physiological role of protein disulphide-isomerase is in the formation of protein disulphide bonds in biosynthesis and assembly.

Further evidence for this role is provided by studies on the distribution of the enzyme in the 17-day chick embryo, where the major extracellular disulphide-containing protein synthesized is procollagen. Protein disulphide-isomerase is present in many tissues of the chick embryo and its specific activity is highest in tissues most active in procollagen synthesis (Harwood & Freedman, 1978; B. E. Brockway, S. J. Forster & R. B. Freedman, unpublished work). The distribution of the enzyme between tissues parallels their activity in procollagen synthesis and is closely similar to the distribution of other enzymes that specifically act on procollagen.

The resolution of protein disulphide-isomerase from other enzymes catalysing related reactions, and the unequivocal demonstration that protein disulphide-isomerase is a distinct enzymic species, will make it easier to establish the cellular role of this enzyme. The purification of the enzyme described here should allow further characterization of its structure and mechanism.

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