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Structural properties of homogeneous protein disulphide-isomerase from bovine liver purified by a rapid high-yielding procedure

Nigel LAMBERT and Robert B. FREEDMAN
Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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1. Protein disulphide-isomerase from bovine liver was purified to homogeneity as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, two-dimensional electrophoresis and *N*-terminal amino acid analysis. The preparative procedure, a modification of that of Carmichael, Morin & Dixon [(1977) *J. Biol. Chem.* **252**, 7163–7167], is much faster and higher-yielding than previous procedures, and the final purified material is of higher specific activity. 2. The enzyme has M_r 57000 as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, both in the presence and in the absence of thiol compounds. Gel-filtration studies on Sephadex G-200 indicate an M_r of 107000, suggesting that the native enzyme is a homodimer with no interchain disulphide bonds. Ultracentrifugation studies give a sedimentation coefficient of 3.5S, implying that the enzyme sediments as the monomer. 3. The isoelectric point, in the presence of 8M-urea, is 4.2, and some microheterogeneity is detectable. 4. The amino acid composition is comparable with previous analyses of this enzyme from bovine liver and of other preparations of thiol:protein disulphide oxidoreductases whose relation to protein disulphide-isomerase has been controversial. The enzyme contains a very high proportion of Glx + Asx residues (27%). The *N*-terminal residue is His. 5. The pure enzyme has a very small carbohydrate content, determined as 0.5–1.0% by the phenol/H_2SO_4 assay. Unless specific steps are taken to remove it, the purified enzyme contains a small amount (5 mol/mol of enzyme) of Triton X-100 carried through the purification.

The enzyme protein disulphide-isomerase (PDI, EC 5.3.4.1) was first discovered by the groups of Anfinsen (Goldberger *et al.*, 1963) and Straub (Venetianer & Straub, 1963) during a search for physiological catalysts of the re-oxidation and re-activation of reduced ribonuclease. Such an enzyme activity was found in microsomal fractions from various tissues (Givol *et al.*, 1964) and was shown to be capable of catalysing the re-activation of several reduced denatured proteins under mildly oxidizing conditions (Goldberger *et al.*, 1964). In these circumstances re-activation occurs in two phases: rapid re-oxidation to give mainly non-native disulphide bonds, followed by slow thiol–disulphide interchange steps in which native disulphide bonds are formed. The enzyme was shown to catalyse the second process (Givol *et al.*, 1964; Venetianer & Straub, 1964). Thus in the presence of a thiol compound the enzyme PDI will convert 'scrambled' ribonuclease (ribonuclease reduced and then re-oxidized in denaturing conditions to give a complex mixture of non-native disulphide bonds) into ribonuclease. The associated appearance of ribonuclease activity is the standard assay for PDI.

In the 1960s PDI was purified from bovine liver by Anfinsen's group and some structural characterization was carried out (De Lorenzo *et al.*, 1966). However, the enzyme has never been subjected to detailed molecular study. Such study is now overdue for a number of reasons. Firstly, studies on the enzyme in several specialized sources, including embryonic connective tissue, mouse spleen and lymphoma cells and developing wheat (which synthesize procollagen, immunoglobulins and wheat storage proteins respectively), have established that the presence and amount of PDI activity are correlated with the synthesis and secretion of disulphide-bonded proteins (Brockway *et al.*, 1980; Roth & Koshland, 1981; Freedman *et al.*, 1983). This supports the original proposal by Anfinsen and

Abbreviations used: PDI, protein disulphide-isomerase; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; SDS, sodium dodecyl sulphate.

colleagues (Epstein *et al.*, 1963) that PDI is involved in the formation of native disulphide bonds during protein biosynthesis and hence is responsible for a major and widespread post-translational modification (Freedman & Hillson, 1980). Secondly, the reaction catalysed by the enzyme is one that is of interest mechanistically. Several enzymes are known to catalyse thiol–disulphide interchange, with disulphide substrates of various relative molecular masses (Freedman, 1979), but in no case have detailed studies of enzyme mechanism been performed. This should be of particular interest in the case of PDI, since the enzyme catalyses multiple disulphide interchange reactions within a macromolecular substrate, leading to formation of a set of disulphide bonds that are usually buried within the most stable folded conformation of the protein, the native state. Finally, there has been a continuing controversy about whether PDI (defined by the ability to reactivate 'scrambled' ribonuclease) is identical with enzyme preparations catalysing other thiol–protein-disulphide oxidoreduction reactions, in particular the reduction of insulin disulphide bonds by reduced glutathione (EC 1.8.4.2). Some comparisons have been made of data in the literature on preparations described as protein disulphide-isomerase, thiol:protein-disulphide oxidoreductase and glutathione:insulin transhydrogenase, but these often involved comparisons between different tissues and species, yielding ambiguous results (Varandani, 1978; Morin *et al.*, 1978).

The lack of molecular information about PDI is partly due to the inadequacy of previous preparative procedures. The conventional method is based on that originally developed by De Lorenzo *et al.* (1966), which involves subcellular fractionation of a liver homogenate, acetone extraction of microsomal fraction, buffer extraction, ammonium sulphate fractionation and three column-chromatography steps. Using this method, Hawkins & Freedman (1976) purified the bovine liver enzyme to a final specific activity of 543 units/g with a yield of 3.7%, and more recently Ohba *et al.* (1981) purified the enzyme from rat liver 130-fold with a yield of 6.5%. In the former case the preparation was not completely homogeneous, and in the latter case additional chromatography steps, which did not improve the specific activity, were necessary to obtain homogeneous enzyme. Hillson & Freedman (1980) incorporated a covalent chromatography step on thiopropyl-Sepharose into the procedure, which gave a homogeneous preparation but did not improve either the yield or the specific activity of the final product.

All the above procedures gave low yields of material after many time-consuming operations. A much speedier approach was introduced by Carmichael *et al.* (1977), who isolated a thiol:protein-disulphide oxidoreductase from bovine liver and showed that it was capable of catalysing re-activation of 'scrambled' ribonuclease (Morin *et al.*, 1978). The method involved initial homogenization in a detergent solution, a heat treatment, ammonium sulphate fractionation and chromatography steps. It gave in good yield a homogeneous preparation (Carmichael *et al.*, 1977; Morin *et al.*, 1978). Roth & Koshland (1981) have used the same method to purify a disulphide-interchange enzyme from mouse liver and spleen, and shown that it was capable of catalysing the reduction of disulphide bonds in insulin and the formation of disulphide bonds in immunoglobulin assembly.

In the present paper we describe a modification of the procedure of Carmichael *et al.* (1977) that gives a very rapid and high-yielding purification of PDI from bovine liver, and we describe some structural characteristics of the enzyme. In the following paper (Lambert & Freedman, 1983) we describe catalytic properties of the homogeneous enzyme and compare them with those of previous preparations of PDI and of preparations described as glutathione:insulin transhydrogenase or thiol:protein-disulphide oxidoreductase.

Experimental

Materials

Ox livers were supplied by Canterbury Abattoir (F.M.C. Meat Ltd.). Sephadex G-200, Sephadex CM-50 and DEAE-Sephacel were supplied by Pharmacia, Cibacron Blue–agarose gel was from Sigma Chemical Co. and Amberlite XAD-2 was from BDH Chemicals. Ampholines were from LKB Produkter, and marker proteins for electrophoresis were supplied by Sigma Chemical Co. and Boehringer. Polyamide sheets for t.l.c. and dansyl-amino acid standards were from BDH Chemicals. Otherwise all materials and chemicals were as previously described (Hillson & Freedman, 1980).

Preparation of PDI

The method was based on that of Carmichael *et al.* (1977), but, since several significant variations were made, the procedure is given in full.

(*a*) *Homogenization in Triton.* Bovine liver from freshly slaughtered animals was freed of connective tissue and cut into small cubes. The diced liver was stored at −20°C in 500g lots. On demand 500g was thawed overnight at 4°C, washed in ice-cold saline (0.9% NaCl) and homogenized in 1 litre of 'homogenizing buffer' [0.1M-sodium phosphate buffer, pH 7.5, containing 5mM-EDTA and 1% (v/v) Triton X-100] in a Waring Blendor at full speed for 4 × 30s bursts. The homogenate was strained through two layers of muslin, centrifuged at 18000*g* for 30min at 4°C, and the pellet discarded.

(*b*) *Heat treatment.* The supernatant was decanted through glass-wool and then placed in a water bath at 70°C with constant stirring. The temperature of the extract was allowed to reach 54°C (±1°C), where it was maintained for 15min. The treated extract was then transferred to an ice bath to cool, and was then centrifuged at 18000*g* for 40min. This centrifugation and all subsequent steps were performed at 4°C.

(*c*) *Ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ was slowly added to the supernatant from the heat denaturation to give 55% saturation. After being stirred for 30min, the material was centrifuged at 18000*g* for 30min, and the pellet discarded. Further $(NH_4)_2SO_4$ was added to give a final saturation of 85%, and the material was centrifuged as before. The supernatant was discarded, and the pellet was taken up in approx. 20ml of 25mM-sodium citrate buffer, pH5.3, and then dialysed overnight against this buffer (2 × 5 litres).

(*d*) *Cation-exchange chromatography.* The dialysed extract was applied to a column (36cm × 6cm) of CM-Sephadex C-50 previously equilibrated with 25mM-sodium citrate buffer, pH5.3, and eluted with the same buffer at a flow rate of 3–4ml/min. Fractions of volume 15ml were collected, and the void protein fractions, which contained PDI activity, were pooled. Protein was precipitated by addition of $(NH_4)_2SO_4$ to give 100% saturation, and the pellet was collected by centrifugation at 18000*g* for 30min. The pellet was dissolved in about 15ml of 20mM-sodium phosphate buffer, pH6.3, and dialysed overnight against the same buffer (2 × 2 litres).

(*e*) *Anion-exchange chromatography.* The dialysed extract was loaded on to a column (35cm × 2cm) of DEAE-Sephacel equilibrated with 20mM-sodium phosphate buffer, pH6.3, and eluted with a linear gradient of 0–0.7M-NaCl in the same buffer, at a flow rate of 20ml/h. Fractions of volume 5ml were collected, and three peaks of protein were detected by absorbance at 280nm. The second peak contained PDI activity; active fractions were pooled, dialysed against 50mM-NH_4HCO_3 and freeze-dried. The freeze-dried enzyme was stored at −20°C. After 9 months' storage the enzyme retained more than 70% of initial activity.

Removal of Triton X-100

Purified isomerase (10ml; 1mg/ml in 50mM-sodium phosphate buffer, pH7.5) was stirred gently with 1g of washed Amberlite XAD-2 resin for 1h at room temperature. The solution was decanted from the beads, a fresh batch of Amberlite was added and the procedure was repeated. Finally, the solution was centrifuged in a bench centrifuge at 3000 rev./min for 10min to remove fines. Control experiments were performed with standard solutions of 0.5% Triton X-100 in the same buffer in the presence and in the absence of bovine serum albumin (1mg/ml).

Assay of PDI activity

The assay method was essentially that of Ibbetson & Freedman (1976), in which the rate of reactivation of 'scrambled' ribonuclease by PDI is monitored by the withdrawal of samples at frequent intervals and their assay for ribonuclease activity by a dual-wavelength spectrophotometric assay. The detailed procedure is given in the following paper (Lambert & Freedman, 1983). The unit of PDI activity is that defined by Ibbetson & Freedman (1976).

Analytical ultracentrifugation

Ultracentrifugation studies were performed in an MSE Centriscan (model 75) analytical ultracentrifuge, with a six-cell titanium rotor (43111-108) and 10mm single-sector cells. Sedimentation-velocity studies were performed at 20°C, with absorbance optics at 280nm; the rotor speed was 50000rev./min. Cells were scanned at 10min intervals with an automated linked recorder. The point of inflexion for each boundary curve was determined, and the distance (*r*) of these points from the centre of the rotor obtained as a function of time.

Gel filtration

Purified PDI (5mg, 5 units) and marker proteins in 25mM-sodium phosphate buffer, pH7.5, were loaded on a column (55cm × 2cm) of Sephadex G-200 equilibrated in the same buffer. The M_r markers were bovine haemoglobin (5mg), *Escherichia coli* alkaline phosphatase (6 units), bovine heart lactate dehydrogenase (62 units), bovine liver catalase (10mg) and horse spleen ferritin (5mg), and the total sample volume was 10ml. The proteins were eluted in this buffer at a flow rate of 10ml/h, and 2ml fractions were collected. The experiment was performed three times at 4°C and twice at 25°C. Ferritin and catalase were detected by absorbance at 280nm, and haemoglobin was detected by absorbance at 410nm. PDI was detected by the standard enzyme assay (indicated above). Lactate dehydrogenase was assayed spectrophotometrically by monitoring NADH absorbance at 340nm. The assay medium (3ml) contained 0.5M-Tris/HCl buffer, pH7.5, 5mM-$MgCl_2$, 2mM-pyruvate, 120μM-NADH and 20–100μl of sample. Assay was performed at 30°C; after 5min equilibration at this temperature, reaction was initiated by addition of pyruvate, and reaction rate was constant for the next 5min. Alkaline phosphatase was assayed spectrophotometrically by monitoring hydrolysis of *p*-nitrophenyl phosphate at 400nm. The assay medium (3ml) contained 1mM substrate in 500mM-Tris/HCl

buffer, pH 8.5. After 5 min equilibration at 30°C, reaction was initiated by the addition of 10–50 μl of sample. Initial rates were constant for up to 5 min. A plot was constructed of marker-protein elution volumes versus $\log M_r$, and the M_r of PDI was obtained by interpolation (Andrews, 1961).

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed in 1.5 mm slab gels with Laemmli (1970) buffers. For routine monitoring of the purification, the acrylamide concentration was 10% (w/v). Samples were denatured by boiling for 10 min in 2% (w/v) SDS either in the presence or in the absence of 5% (v/v) 2-mercaptoethanol (see below). For estimation of the M_r of purified PDI, linear-gradient gels (7½–16½%, w/v) were used. The marker proteins were: soya-bean trypsin inhibitor, ovalbumin, catalase, bovine serum albumin and phosphorylase *b*. Gels were stained with Coomassie Brilliant Blue R-250.

Isoelectric focusing

Isoelectric focusing was performed in cylindrical gels (0.25 cm × 12 cm). Gels contained 4% (w/v) polyacrylamide, 8 M-urea, pH 4–6 Ampholines (1.6%, w/v) and pH 3.5–10 Ampholines (0.4%, w/v). Samples were applied at the basic end, and control gels were focused concurrently. After the focusing, proteins were detected by staining with Bromophenol Blue (0.4%), and the pH gradient of control gels was obtained by cutting 5 mm slices and allowing them to equilibrate in water.

Two-dimensional electrophoresis

The method was essentially that of O'Farrell (1975). Isoelectric focusing was performed as described above. The cylindrical gels were then layered on to linear-gradient polyacrylamide gels (7½–16½%, w/v), and SDS/polyacrylamide-gel electrophoresis was performed as described above. Gels were stained with Coomassie Brilliant Blue R-250.

Amino acid analysis

This was performed independently at the Chester Beatty Institute of Cancer Research, London, U.K., by standard methods.

Identification of N-terminal amino acid

The method was that of Hartley (1970). A sample of enzyme (1–5 nmol) was incubated with 10 μl of 0.2 M-$NaHCO_3$, 10 μl of dansyl chloride (2.5 mg/ml in acetone) and 10 μl of water in a stoppered tube at 30°C for 3 h. After evaporation to dryness in a stream of N_2, the sample was hydrolysed with 6 M-HCl (50 μl) in a sealed ignition tube at 100°C for 16 h. The sample was then placed over NaOH pellets in an evacuated desiccator, and the dry residue was dissolved in 100 μl of acetone/acetic acid (3:2, v/v). Samples (10 μl) were spotted on to one corner of polyamide thin-layer sheets (10 cm × 10 cm) and separated in two dimensions. For the first dimension the solvent was water/formic acid (200:3, v/v), and for the second either benzene/acetic acid (9:1, v/v) alone or benzene/acetic acid followed by ethyl acetate/methanol/acetic acid (20:1:1, by vol.). Authentic dansyl-amino acids were run concurrently with the sample on the other side of the polyamide sheets; dansylated amino acids were observed under u.v. light.

Determination of carbohydrate content

The method of Dubois *et al.* (1956) was used to determine the total carbohydrate content of the purified enzyme. A 50 μl volume of 80% (w/v) phenol was added to 2 ml of sample in 25 mM-sodium phosphate buffer, pH 7.5. Conc. H_2SO_4 (5 ml) was rapidly added, and the sample was left at room temperature for 10 min before incubation at 30°C for 15 min. After a further 30 min at room temperature, the absorbance was read at 490 nm. Calibration curves were constructed with mannose and galactose as standards. The relationship between A_{490} and sugar content was linear over the range 0–100 μg for these sugars. In agreement with the published literature (Dubois *et al.*, 1956), mannose gave higher absorbance than galactose, under the conditions used. Samples of ovalbumin with a known carbohydrate content of 2% and of bovine serum albumin (no carbohydrate) were tested to check the assay. Triton X-100 (0.1%) was shown not to interfere with the determination.

Determination of protein

The method was that of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Results and discussion

Purification of bovine liver PDI

The procedure described in the Experimental section involves five steps and yields 200 mg of homogeneous enzyme from 1 kg of liver. Table 1 gives data on the purification and yield through one typical preparation and summarizes the final results for five preparations.

The first step involves homogenization of the tissue in the presence of Triton X-100 to solubilize the enzyme. The detergent/protein ratio used in this study was higher than that used by Carmichael *et al.* (1977) (2 ml of 1% Triton/g of liver rather than 0.5 ml/g), ensuring a more efficient dispersion and solubilization. Only a small amount of enzyme activity is solubilized when the tissue is homogenized in buffers in the absence of detergents (Hawkins & Freedman, 1976). The enzyme is associated with the endoplasmic reticulum (Givol

Table 1. *Purification of PDI from bovine liver (1 kg)*

For experimental details see the text. In the last row means ± S.D. for five preparations of the final extract are given in parentheses.

	Protein (g)	PDI activity (units)	Specific activity (units/g)	Yield (%)
Homogenate	191	770	4.03	100
18000 *g* supernatant	137	750	5.47	97
Heat-treatment supernatant	31.2	624	20.0	81
55–85%-satn.-$(NH_4)_2SO_4$ fraction	9.16	414	45.2	54
CM-Sephadex, pH 5.3 eluate	1.45	262	181	34
DEAE-Sephacel, pH 6.3 eluate	0.22	206	936	27
	(0.19 ± 0.04)	(197 ± 25)	(1001 ± 136)	(25 ± 6)

et al., 1964), and it has previously been observed that treatment of microsomal fractions with detergents leads to solubilization of the enzyme and considerable activation (Ohba *et al.*, 1977; Freedman *et al.*, 1978). In more recent work we have shown that the enzyme is completely latent in carefully prepared rat liver microsomal fraction, but can be activated by sonication, detergent treatment etc. (Freedman *et al.*, 1983). The presence of detergent in the first stages of purification leads to a slight underestimate of the total amount of enzyme units solubilized, since small amounts of detergent are carried over into the assay incubation and low concentrations of detergents are known to inhibit the enzyme activity both in microsomal fractions and in purified preparations (Hawkins & Freedman, 1976; Freedman *et al.*, 1978).

The second stage of the procedure is a heat treatment, which exploits the enzyme's marked thermal stability. Only 20% of total PDI activity is lost during incubation of the enzyme for 15 min at 54°C (Table 1), and the enzyme is 4-fold purified by this step. After an ammonium sulphate fractionation of the heat-stable extract, the final stages of the procedure are two ion-exchange chromatography steps. Use of the cation-exchanger CM-Sephadex C-50 at the low pH of 5.3 ensures that most of the proteins in the extract are retarded while highly acidic proteins such as PDI are eluted in the void volume. This convenient step gives a significant purification (4-fold). After CM-Sephadex chromatography the preparation contained two major components, one of M_r 57000 and one of M_r 68000, which was assumed to be serum albumin.

The final step was chromatography on DEAE-Sephacel at pH 6.3, a pH at which serum albumin is nearly net neutral whereas PDI is significantly negatively charged. Proteins were eluted from DEAE-Sephacel with a gradient of NaCl, and Fig. 1 shows the profile of a typical elution. Three peaks of protein were detected, and PDI activity was found only in the second peak. The first peak was predominantly a protein of M_r 68000 and the third peak comprised several proteins of M_r 40000–50000. The active fractions in the second peak were pooled and found to be homogeneous by several electrophoretic criteria (see Figs. 2–4). This chromatographic step was preferred to the anion-exchange chromatography step described by Carmichael *et al.* (1977), which used DEAE-Sephadex at pH 7.8, a pH above the pI of both PDI and serum albumin. This step also gave better resolution of PDI and serum albumin than was obtained by dye-affinity chromatography on Cibacron Blue–agarose (Travis *et al.*, 1976) (results not shown).

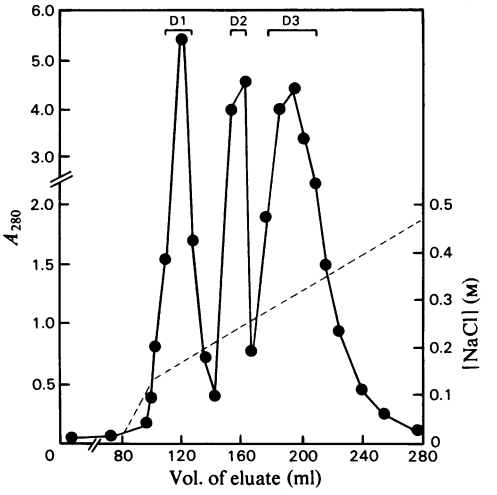

Fig. 1. *Elution of PDI from DEAE-Sephacel column*

See the Experimental section for full details. ●—●, A_{280}; ----, concn. of NaCl. D1, D2 and D3 represent the fractions pooled and retained for analysis.

The purification procedure is greatly improved over the previous method derived from De Lorenzo *et al.* (1966), which was very laborious and gave very low yields of enzyme (Hawkins & Freedman, 1976; Hillson & Freedman, 1980; Ohba *et al.*,

1981). By the method described above, two 500g batches of liver can be processed in less than 2 weeks to yield approx. 200mg of homogeneous enzyme, with a yield of 25–30%. The poor yields of the previous methods arose primarily in the preparation of microsomal fractions. Losses of approx. 70% (Hawkins & Freedman, 1976) or 50% (Ohba *et al.*, 1981) of PDI activity were observed in the isolation of the microsomal fraction. A large proportion of the endoplasmic reticulum in liver is closely associated with mitochondria, and remains stably associated with it on homogenization to give the so-called 'rapidly sedimenting endoplasmic reticulum', which can be recovered from nuclear and mitochondrial fractions (Shore & Tata, 1977; Meier *et al.*, 1978; Pickett *et al.*, 1980). Preparation of the liver homogenate in detergent to solubilize the PDI by-passed this lengthy and wasteful subcellular fractionation. In the conventional preparation the microsomal fraction is subsequently extracted with acetone and then with aqueous buffer, a procedure that is less efficient than detergent in solubilizing the enzyme, and more likely to produce inactivation.

The specific activity of the final homogeneous material is about 2-fold higher than those reported for previous preparations from this laboratory (Hawkins & Freedman, 1976; Hillson & Freedman, 1980). The material is approximately 250-fold purified relative to the initial homogenate; this is actually a lower degree of purification than those reported previously, but in previous work purification was referred to the initial homogenate in an iso-osmotic sucrose buffer, and so the initial activity was very significantly underestimated because of the latency of the enzyme (see above). The 250-fold purification found here for the homogeneous enzyme implies that PDI constitutes about 0.4% of extractable liver protein. This is consistent with estimates made by immunological techniques on the amounts of PDI in rat and mouse liver, which gave results of 0.37% (Ohba *et al.*, 1981) and 0.35% (Roth & Koshland, 1981) respectively.

Presence of Triton X-100 in the purified enzyme

Direct assay (Garewal, 1973) showed the presence of approx. 0.03mg of Triton/mg of protein in the PDI preparation. Amberlite XAD-2 is claimed to remove Triton X-100 from proteins (Cheetham, 1979), and control experiments with 0.5% (v/v) Triton both in the absence and in the presence of bovine serum albumin (1mg/ml) confirmed that the resin removed all the detergent as determined by absorbance at 280nm and direct assay. Experiments performed on Amberlite-treated PDI showed that there were no significant structural or kinetic differences between this material and that which had not been subjected to Amberlite treatment. Thus the treatment to remove Triton X-100 was not introduced as a routine part of the purification procedure, and most of the results reported in the present paper are from studies on untreated material.

Physical properties of the pure enzyme

Isoelectric focusing of the purified enzyme on shallow pH gradients in the presence of 8M-urea gave a single band of protein with an isoelectric point of 4.2 ± 0.1 (Fig. 2). At high loading some microheterogeneity could be seen within the protein band; this is likely to have arisen as an artifact from deamidation, carbamoylation or the formation of isomers differing in thiol–disulphide status (Wallevik, 1976; Carmichael *et al.*, 1979), since the apparent extent of microheterogeneity increased with sample loading (Cann, 1979; Gianazza & Righetti, 1980).

Analysis of the purified material by SDS/polyacrylamide-gel electrophoresis also showed one protein band, which migrated with M_r 57000 ± 2000 both in the presence and in the absence of 2-mercaptoethanol, indicating the absence of interchain disulphide bridges (Fig. 3). Some high-M_r material could be detected in the absence of reductant. This is likely to result as an artifact from oxidation by molecular oxygen, since no alkylating agent was included to block free thiol groups and the intensity of this band increased with storage. Combination of isoelectric focusing and SDS/polyacrylamide-gel electrophoresis in a two-dimensional electrophoresis procedure confirmed the homogeneity of the purified material (Fig. 4).

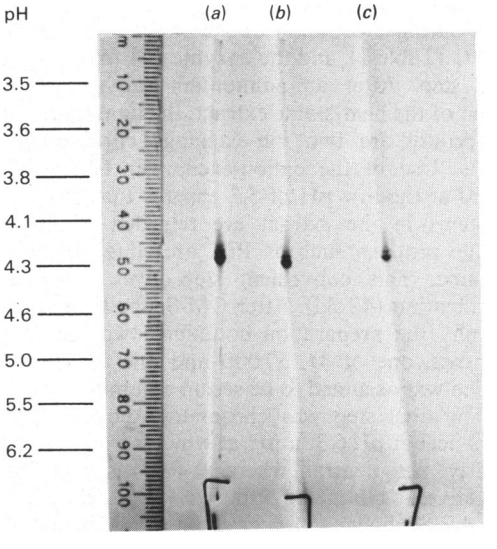

Fig. 2. *Isoelectric focusing of purified PDI*
See the Experimental section for full details. The gels were loaded with (*a*) 20μg, (*b*) 10μg and (*c*) 5μg of purified PDI.

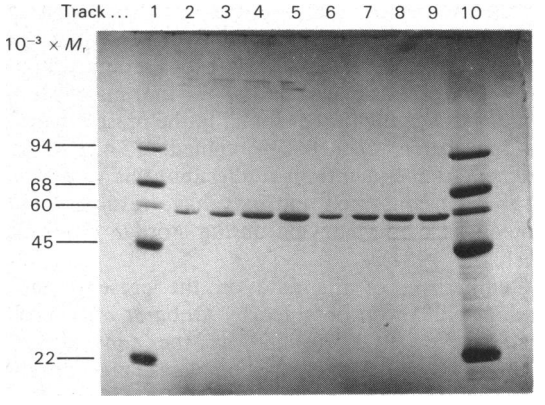

Fig. 3. *SDS/polyacrylamide-gel electrophoresis of purified PDI*

See the Experimental section for full details. Tracks 1 and 10, M_r standards; tracks 2–5, purified enzyme in the absence of 2-mercaptoethanol (2, 4, 6 and 8 μg of protein respectively); tracks 6–9, as for tracks 2–5 but in the presence of 2% (v/v) 2-mercaptoethanol.

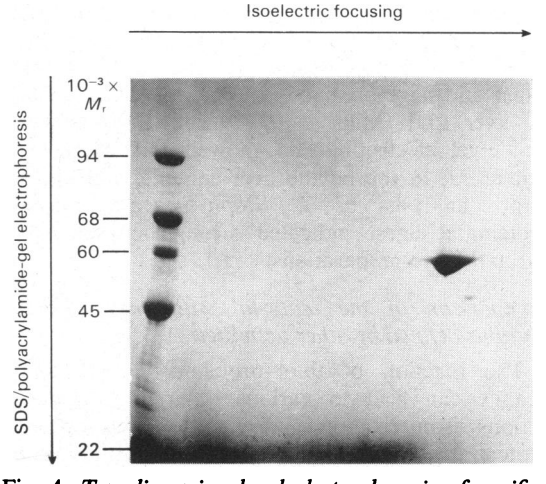

Fig. 4. *Two-dimensional gel electrophoresis of purified PDI*

A 10 μg portion of pure enzyme was loaded and subjected to two-dimensional electrophoresis as described in the Experimental section.

The purified enzyme was subjected to gel filtration on Sephadex G-200, and it was eluted as a symmetrical peak. The peak of maximum activity was at a volume between that of lactate dehydrogenase (M_r 136000) and alkaline phosphatase (M_r 86000). By comparison with standards ranging in M_r from 64000 to 450000, an M_r value of 107000 $\pm$ 5000 was obtained for the native

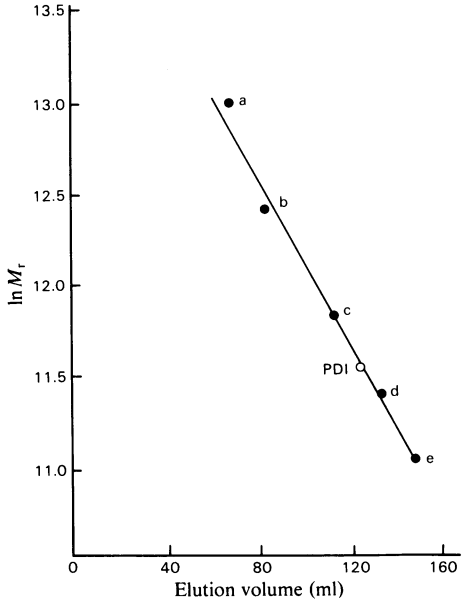

Fig. 5. *Estimation of M_r of purified PDI by gel filtration of Sephadex G-200*

See the Experimental section for full details. Markers: a, ferritin; b, catalase; c, lactate dehydrogenase; d, *E. coli* alkaline phosphatase; e, haemoglobin. The experiment was performed three times at 4°C and once at 25°C with identical results; the experiment at 25°C was for comparison with data from sedimentation-velocity experiments.

enzyme in solution (Fig. 5), both at 4°C and at 25°C. This result, together with the polypeptide M_r determined by SDS/polyacrylamide-gel electrophoresis, suggests that the enzyme is present in solution as a homodimer.

Analytical-ultracentrifugation experiments on the purified enzyme with absorbance optics were hindered by the presence of Triton X-100, which absorbs at 280nm. Hence sedimentation-velocity studies on protein disulphide-isomerase were performed on Amberlite-treated material. This gave a sedimentation coefficient (s_{20}) of 3.5 $\pm$ 0.4S for all concentrations of protein employed between 0.5 and 2.0mg/ml, which is more consistent with an M_r value of 57000 than with one of 107000, suggesting that the enzyme exists as a monomer at the hydrostatic pressures experienced in the ultracentrifuge.

Chemical composition of the pure enzyme

The amino acid composition of the purified enzyme is given in Table 2. Tryptophan analysis was not performed. The analysis revealed a high proportion of Glx and Asx, consistent with the acidic isoelectric point of the enzyme. A single *N*-terminal

Table 2. *Amino acid composition of bovine liver PDI*

Amino acid	Composition (residues/100 residues)
Lys	10.97
His	2.37
Arg	2.32
Asx	12.18
Thr	4.57
Ser	4.88
Glx	14.98
Pro	4.47
Gly	6.57
Ala	8.99
Val	5.26
Met	0.86
Ile	3.84
Leu	8.72
Tyr	2.26
Phe	6.26
Cys	0.51*
Total	100.01

* Value for cysteine is probably an underestimate.

amino acid, His, was found by the conventional dansylation procedure. The finding of only a single *N*-terminal residue is further evidence that the preparation is homogeneous and consists of identical polypeptide chains.

A crude estimate of the carbohydrate content of the purified enzyme was obtained by the phenol/H_2SO_4 method of Dubois *et al.* (1956). Different sugars give different sensitivities in this assay, and separate standard curves were therefore constructed with mannose and galactose. The method gave satisfactory results with these standards and with proteins of known carbohydrate content (see the Experimental section). Purified PDI was found to have a low but detectable carbohydrate content, estimated at 0.5–1% by comparison with the above standards. This could be contaminating carbohydrate components derived from the preparative chromatography columns, but it may represent genuine covalently bound carbohydrate.

Comparison of the material with previous preparations of liver PDI

The material described above is very similar in properties to previous samples of PDI prepared in this laboratory by different methods. The polypeptide M_r and isoelectric point agree with those found by Hawkins & Freedman (1976), and the M_r and amino acid composition agree with those found by Hillson & Freedman (1980, and unpublished work). There is little doubt that this new preparation yields material essentially identical with that obtained by previous methods, but more homogeneous and more active. The material is also probably identical with the first pure samples of PDI, obtained by De Lorenzo *et al.* (1966). A sedimentation coefficient of 3.26 S was quoted for that material, in good agreement with that found in the present work. De Lorenzo *et al.* (1966) obtained M_r 42000 by sedimentation-equilibrium studies, but this value has never been confirmed and may have resulted from proteolysis of the enzyme during storage (Roth & Koshland, 1981).

Comparison of the data on the present preparation with those obtained by Ohba *et al.* (1981) for rat liver PDI, purified by the conventional method, suggests that the enzymes are closely homologous. Ohba *et al.* (1981) obtained a polypeptide M_r of 52000 by SDS/polyacrylamide-gel electrophoresis and a much larger value by gel filtration. They estimated a value of 90000–95000, but the choice of gel-filtration markers did not make an accurate assessment possible. Ohba *et al.* (1981) proposed that the purified rat liver enzyme existed in solution as a homodimer. The purified rat liver PDI obtained by that group did not contain significant quantities of carbohydrate, as determined by g.l.c. of a hydrolysate (T. Omura, personal communication). All these findings agree with those reported in the present paper. We have in fact applied to rat liver the method of purification described in the present paper and have obtained a homogeneous sample of rat liver PDI (Mills *et al.*, 1983). SDS/polyacrylamide-gel electrophoresis showed this to have a similar M_r to the bovine liver enzyme, and 'fingerprint' analysis of a *Staphylococcus aureus*-proteinase digest indicated strong homology between the two preparations.

Comparison of the material with enzyme preparations defined by other activities

The reaction of thiol–protein-disulphide interchange can lead to various different overall reactions: isomerization of disulphide bonds within a protein, as in the assay of PDI, formation of interchain disulphide bonds, reduction of protein disulphides etc. (for review see Freedman, 1979). Thus an enzyme catalysing thiol–protein-disulphide interchange can, in theory, have a number of different observable activities and can be assayed in a number of ways, depending on its specificity. This has led to the use of a number of different names and to controversy as to whether different observable activities arise from single or multiple enzyme species.

The material described in the present paper has been referred to as protein disulphide-isomerase and has been assayed throughout by its ability to catalyse re-activation of 'scrambled' ribonuclease. The method of purification is based on one developed by Carmichael *et al.* (1977) and used for the purification of a material described by them as

thiol:protein-disulphide oxidoreductase, and assayed by its ability to catalyse the reduction of insulin disulphide bonds by reduced glutathione. The obvious question is whether our material is identical with that studied by Carmichael *et al.* (1977), who found an M_r value of 60000–62000 by SDS/polyacrylamide-gel electrophoresis and pI 4.1–4.4, in good agreement with our results. They obtained a value of 60000 for the M_r by sedimentation equilibrium, in agreement with that found by SDS/polyacrylamide-gel electrophoresis, and a considerably higher value by gel filtration. Carmichael *et al.* (1977) found their preparation to have a remarkably high carbohydrate content (12% by wt.), but this finding has subsequently been revised to approx. 1% (J. E. Dixon, personal communication).

The method of Carmichael *et al.* (1977) was used by Roth & Koshland (1981) to prepare an enzyme from mouse liver and spleen that catalysed disulphide interchange. They assayed their preparation by the glutathione–insulin oxidoreduction reaction, but showed that the material also catalysed other disulphide-interchange reactions, including the formation of disulphide bonds in immunoglobulins; they referred to the enzyme as disulphide-interchange enzyme. This preparation, like ours and like that of Carmichael *et al.* (1977), had an M_r of close to 60000 by SDS/polyacrylamide-gel electrophoresis and pI in the range 4.0–4.5.

Extensive work has been carried out by Varandani and colleagues on enzymes catalysing the glutathione–insulin oxidoreduction reaction and termed by them glutathione:insulin transhydrogenase (for review see Varandani, 1978). The best-characterized preparation is from bovine pancreas. This material has M_r 60000 by SDS/polyacrylamide-gel electrophoresis, pI 4.5–4.9 and a sedimentation coefficient of 3.27S; it has a low carbohydrate content (1.6%), and *N*-terminal amino acid analysis showed the presence of three *N*-terminal residues, Leu, Lys and Val. An analogous preparation from rat liver (Ansorge *et al.*, 1973*b*) also has M_r 60000 by SDS/polyacrylamide-gel electrophoresis and apparently a native M_r of 50000–55000 estimated by gel filtration.

Clearly there are some striking structural similarities between all these preparations, but also some apparent discrepancies. Of the data available, amino acid compositions provide the best opportunity for a quantitative measure of their relatedness (Cornish-Bowden, 1981). Three measures have been proposed to assess quantitatively the relatedness of proteins from amino acid composition data; the difference index *DI*, the compositional divergence *D*, and a squared index $S\Delta Q$, which is equal to $(100D)^2$ (for review see Cornish-Bowden, 1980). These values are small for closely related proteins and increase rapidly with divergence; for polypeptides of any given size, upper-limit values for each of these parameters can be given for related polypeptides. For polypeptides of about 500 residues the relevant 'critical values' for *DI*, $S\Delta Q$ and *D* are 6.47, 16.8 and 0.041 respectively; where comparison between two sets of composition data gives values below these, there is negligible probability that the proteins are not related (Cornish-Bowden, 1980).

We have compared the amino acid composition data for our preparation of bovine liver PDI (Table 2) with each of five other sets of data: for bovine liver PDI (De Lorenzo *et al.*, 1966), rat liver glutathione:insulin transhydrogenase (Ansorge *et al.*, 1973*a*), bovine pancreas glutathione:insulin transhydrogenase (Varandani, 1974), bovine liver thiol:protein-disulphide oxidoreductase (Morin *et al.*, 1978) and mouse liver disulphide-interchange enzyme (Roth & Koshland, 1981). The comparisons give values for *DI* between 2.49 and 4.23, for $S\Delta Q$ between 1.93 and 8.31 and for *D* of between 0.014 and 0.029. Thus by these criteria the proteins are almost certainly related in sequence. [By contrast, the values for a comparison between our data and that for a thiol oxidase (Janolino & Swaisgood, 1975) from bovine milk were 16.4, 104 and 0.102 respectively.]

A final conclusion on the relatedness of these preparations requires parallel studies on a broad range of properties. The structural data reported here and the catalytic properties presented in the following paper (Lambert & Freedman, 1983) provide strong evidence that the preparations isolated by various methods from various sources and termed PDI, disulphide-interchange enzyme, glutathione:insulin transhydrogenase or thiol:protein-disulphide oxidoreductase are identical or homologous proteins. This conclusion has also been reached from immunological studies on preparations made in several laboratories (S. Bjelland, personal communication).

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