Bovine liver thiol–protein disulphide oxidoreductases

An alternative method for differential purification and resolution of protein disulphide-isomerase and glutathione–insulin transhydrogenase

David A. HILLSON* and Robert B. FREEDMAN

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

(Received 10 March 1980/Accepted 30 June 1980)

1. Protein disulphide–isomerase (EC 5.3.4.1) and glutathione–insulin transhydrogenase (EC 1.8.4.2) activities in bovine liver were studied in parallel during purification of ‘thiol–protein disulphide oxidoreductase’ by the procedure of Carmichael, Morin & Dixon [(1977) *J. Biol. Chem.* **252**, 7163–7167]. The two activities showed no quantitative co-purification and were partially resolved by $(\text{NH}_4)_2\text{SO}_4$ precipitation, indicating that distinct enzymes are present. 2. Protein disulphide-isomerase was purified by a relatively rapid method involving a combination of the early stages of the Carmichael procedure and covalent chromatography, with a new stepwise elution procedure. Ion-exchange chromatography yields a homogeneous preparation of mol.wt. 57000. 3. The relationship between protein disulphide-isomerase, glutathione–insulin transhydrogenase and ‘thiol–protein disulphide oxidoreductase’ is discussed.

There has been considerable controversy over the relationship between the two related thiol–protein disulphide oxidoreductase activities, protein disulphide-isomerase (EC 5.3.4.1) and glutathione–insulin transhydrogenase (EC 1.8.4.2) (Ansorge *et al.*, 1973a,b; Varandani, 1974, 1978; Freedman, 1979). Circumstantial evidence has been presented suggesting that two distinct enzymes are responsible for the measured activities (Hawkins & Freedman, 1976; Ibbetson & Freedman, 1976), and recent work has confirmed this by resolving the two activities from a common source (Hillson & Freedman, 1979, 1980). During these studies, differential purification has been used as a criterion for the non-identity of protein disulphide-isomerase with glutathione–insulin transhydrogenase, and the importance of parallel quantitative experimentation has been stressed.

The purification from bovine liver of a homogeneous preparation catalysing thiol–protein disulphide oxidoreductions has been described by Dixon and co-workers (Carmichael *et al.*, 1977, 1979; Morin *et al.*, 1978). The purified material

apparently has both protein disulphide-isomerase and glutathione–insulin transhydrogenase activities, although quantitative details on the degree of purification of the two activities have not been given. The purification procedure is rapid and convenient, involving homogenization in the presence of Triton X-100, heat denaturation, $(\text{NH}_4)_2\text{SO}_4$ fractionation, ion-exchange chromatography and gel filtration. It was decided to follow protein disulphide-isomerase and glutathione–insulin transhydrogenase activities in parallel in this procedure, both to examine their co-purification behaviour and to clarify their relationship with the enzyme activity purified by Carmichael *et al.* (1977) and designated ‘thiol–protein disulphide oxidoreductase’.

Previous work has shown that covalent chromatography on matrix-bound disulphides can resolve protein disulphide-isomerase from glutathione–insulin transhydrogenase when a sequential elution procedure is used (Hillson & Freedman, 1979, 1980). Covalent chromatography, like affinity chromatography, is a highly specific technique, and can be applied most powerfully at an early stage in a purification schedule. For this reason, the procedure of Carmichael *et al.* (1977) and covalent chromatography with sequential elution (Hillson & Freedman, 1979) were combined in an attempt to produce a simple rapid purification of protein disulphide-isomerase.

Abbreviations used: SDS, sodium dodecyl sulphate; GSH, reduced glutathione.

* Present address: Biophysics Laboratory, Portsmouth Polytechnic, White Swan Road, Portsmouth PO1 2DT, U.K.

Materials and methods

Materials

All materials, and methods except where otherwise stated, were as in the preceding paper (Hillson & Freedman, 1980).

Purification procedure

Fresh bovine liver (500 g) was subjected to the procedure described by Carmichael *et al.* (1977) for the preparation of 'thiol-protein disulphide oxidoreductase'. Washed tissue was homogenized in 1% Triton X-100 at pH 7.5. After centrifugation and filtration, the filtered supernatant was heated at 53°C for 15 min. The supernatant after heat denaturation was fractionated by $(\text{NH}_4)_2\text{SO}_4$ precipitation, and proteins precipitated between 60 and 85% saturation were further fractionated by chromatography on CM-Sephadex C-50, Sephadex G-200 and DEAE-Sephadex A-50.

Samples of material at various stages of purification were retained and dialysed into TKM buffer, pH 7.5 (50 mM-Tris/25 mM-KCl/5 mM-MgCl₂, adjusted to pH 7.5 with HCl), before assays.

Covalent chromatography

Partially purified material [the 60–85%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction] was fractionated by covalent chromatography on thiopropyl-Sepharose 6B (Pharmacia), by using the sequential elution procedure described previously (Hillson & Freedman, 1979). A sample of protein (100–200 mg) was reduced with 0.1 mM-dithiothreitol at 30°C for 30 min; at this stage a considerable amount of protein was precipitated out (estimated at 35–40%

of the sample), and was discarded. The reduced sample was eluted from a column (2.5 cm × 25 cm) of Sephadex G-25 in TKM/EDTA/NaCl buffer, pH 7.5 (i.e. TKM buffer containing 1.25 mM-EDTA and 0.1 M-NaCl), and precipitated protein was retarded on the surface of the column. Reduced protein was loaded on to the thiopropyl-Sepharose 6B (50 g wet wt. of gel) by incubation at 25°C for 16 h with gentle shaking, after which time the mixture was poured into a column (2.5 cm × 10 cm) and equilibrated to 4°C. Unbound protein (fraction W) was washed from the column with TKM/EDTA/NaCl buffer, followed by the three successive reducing steps containing 20 mM-L-cysteine, 50 mM-GSH and 20 mM-dithiothreitol (each in TKM/EDTA/NaCl buffer) respectively. Fraction I (displaced by L-cysteine), fraction II (displaced by GSH) and fraction III (displaced by dithiothreitol) were reduced with 5 mM-dithiothreitol at 25°C for 30 min, and then dialysed extensively against TKM buffer, pH 7.5, at 4°C before assays of protein concentration and enzymic activities.

Results

Differential purification of protein disulphide-isomerase and glutathione-insulin transhydrogenase activities

Protein disulphide-isomerase and glutathione-insulin transhydrogenase activities were measured in parallel at the various stages of purification during the procedure of Carmichael *et al.* (1977). Specific activities were calculated and the results are expressed as degree of purification relative to the homogenate (Table 1). Results are given for two

Table 1. *Differential purification of protein disulphide-isomerase and glutathione-insulin transhydrogenase by the method of Carmichael et al. (1977)*

Results shown are from two separate preparative experiments; preparation 2 was only taken to the stage of $(\text{NH}_4)_2\text{SO}_4$ precipitation. Specific activities are expressed in units/g of protein. Values in parentheses are purification factors relative to the homogenate. N.D., Not detectable.

Fraction	Specific activity (purification factor)			
	Protein disulphide-isomerase		Glutathione-insulin transhydrogenase	
	Prep. 1	Prep. 2	Prep. 1	Prep. 2
Homogenate	1.84 (1)	1.60 (1)	0.25 (1)	0.23 (1)
Heat-denatured supernatant	6.64 (3.6)	4.77 (3.0)	0.39 (1.5)	0.61 (2.7)
$(\text{NH}_4)_2\text{SO}_4$ fractions:				
10–50%-satn.	0.04 (0.02)	0.44 (0.3)	0.05 (0.2)	0.17 (0.8)
50–60%-satn.	2.38 (1.3)	2.07 (1.3)	0.92 (3.6)	0.94 (4.2)
60–85%-satn.	8.00 (4.4)	11.90 (7.4)	0.61 (2.4)	0.83 (3.7)
CM-Sephadex eluate	44.5 (24.2)	—	N.D.	—
Sephadex G-200 eluate	113 (61.3)	—	0.68 (2.7)	—
DEAE-Sephadex eluate	68.1 (37.0)	—	N.D.	—

preparative experiments, the second of which was taken only to the $(\text{NH}_4)_2\text{SO}_4$ fractionation stage. The two activities did not co-purify at any stage of the procedure and were partially resolved by $(\text{NH}_4)_2\text{SO}_4$ fractionation. In our hands glutathione-insulin transhydrogenase activity is reproducibly found mainly in the 50–60%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction, whereas protein disulphide-isomerase activity is concentrated in the 60–85%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction. After ion-exchange chromatography and gel filtration, the resultant material is significantly more purified in protein disulphide-isomerase than in glutathione-insulin transhydrogenase activity.

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

The $(\text{NH}_4)_2\text{SO}_4$ fraction that was rich in protein disulphide-isomerase (the 60–85%-satn. fraction) was subjected to covalent chromatography with the sequential elution procedure. The results of three runs are shown in Table 2, which shows that the two activities are resolved from one another; the behaviour of the activities is qualitatively the same as in covalent chromatography of another bovine liver preparation partially purified by another method (Hillson & Freedman, 1979, 1980). Thus fraction I (displaced by L-cysteine) had only protein disulphide-isomerase activity, whereas glutathione-insulin transhydrogenase activity was only found in fraction III (displaced by dithiothreitol); fraction II (displaced by GSH) showed no detectable activities.

Analysis of the fractions by SDS/polyacrylamide-gel electrophoresis showed that fraction I contained two bands corresponding to mol.wts. of 56 000–58 000 and 68 000–70 000; fraction III showed one major band corresponding to a mol.wt. of 34 000–37 000. The starting material [60–85%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction] showed 13–15 bands, and the material unbound to the column (fraction W) showed the same bands, but the intensities of bands corresponding to mol.wts. of 68 000–70 000,

56 000–58 000 and 34 000–37 000 were considerably diminished. These results are consistent with previous conclusions that in bovine liver the protein responsible for protein disulphide-isomerase activity has a mol.wt. of 57 000 whereas that responsible for glutathione-insulin transhydrogenase activity has a mol.wt. of 37 000 (Hillson & Freedman, 1980).

Further purification by ion-exchange chromatography

Combination of the early stages of the Carmichael *et al.* (1977) procedure with covalent chromatography produced in three steps [heat denaturation, $(\text{NH}_4)_2\text{SO}_4$ fractionation and covalent chromatography] a preparation of protein disulphide-isomerase free from glutathione-insulin transhydrogenase activity (namely fraction I). However, this material was not homogeneous by SDS/polyacrylamide-gel electrophoresis, and so an attempt was made at further purification by ion-exchange chromatography on DEAE-Sephadex A-50, with elution with NaCl steps of zero, 0.25, 0.51 and 0.7 M in 20 mM-phosphate buffer, pH 6.3. Three fractions were obtained, designated fraction D1 (eluted in the void volume at zero NaCl), fraction D2 (eluted at 0.25 M-NaCl) and fraction D3 (eluted at 0.35 M-NaCl); these were assayed for protein disulphide-isomerase activity and analysed by SDS/polyacrylamide-gel electrophoresis. The results are shown in Table 3. Protein disulphide-isomerase activity was found mainly in fraction D3, which showed a major band corresponding to a mol.wt. of 57 000 on gels, with only a trace of a 70 000–71 000-mol.wt. band detectable at high loadings. The specific activity of this fraction is not as high as that found in corresponding purified fractions obtained after another longer purification procedure (Hillson & Freedman, 1980), probably as a result of the detergent and heat treatments at early stages of this procedure. However, both procedures result in preparation of a protein species that has high protein disulphide-isomerase activity, little or no gluta-

Table 2. Resolution by covalent chromatography of enzyme activities in material partially purified by the method of Carmichael *et al.* (1977)

Results shown are from three small-scale covalent chromatography runs with the same starting material. Specific activities are expressed in units/g of protein. Values in parentheses are purification factors relative to the 60–85%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction. N.D., Not detectable.

Fraction	Protein disulphide-isomerase specific activity			Glutathione-insulin transhydrogenase specific activity		
60–85%-satn. $(\text{NH}_4)_2\text{SO}_4$ fraction		11.91 (1)			0.83 (1)	
Fraction I	27.9 (2.3)	23.0 (1.9)	25.2 (2.2)	N.D.	N.D.	N.D.
Fraction II	1.11 (0.1)	4.02 (0.3)	N.D.	0.16 (0.2)	N.D.	N.D.
Fraction III	4.31 (0.4)	6.84 (0.6)	0.55 (0.1)	0.50 (0.6)	0.66 (0.8)	0.81 (1.0)

Table 3. *Further purification of the active fraction from covalent chromatography*

Fraction I from a covalent chromatography run (see Table 2) was applied to DEAE-Sephadex A-50 at pH 6.3 and fractionated by a stepwise elution with increasing concentrations of NaCl. For experimental details see the text. N.D., Not detectable.

	Specific activity (units/g)	Purification factor	Yield (%)	Protein yield (%)	Mol.wt. of bands on SDS/ polyacrylamide-gel electrophoresis
Fraction I	23.03	(1)	(100)	(100)	68 000–70 000 56 000–58 000
Fraction D1	N.D.	—	0	27.8	Indistinct staining
Fraction D2	1.16	0.05	4.2	35.4	70 000–71 000
Fraction D3	63.71	2.77	55.7	10.5	56 000–57 000 (70 000–71 000, faint)

thione–insulin transhydrogenase activity and a mol.wt. on SDS/polyacrylamide-gel electrophoresis of 57 000.

Discussion

Differential purification of the two measured activities, protein disulphide-isomerase and glutathione–insulin transhydrogenase, when assayed in parallel throughout a purification procedure, strongly supports the view that more than one distinct enzymic species is responsible for these activities. This conclusion has been drawn from previous studies on the activities in bovine liver, with the conventional procedure for purification of protein disulphide-isomerase (Hawkins & Freedman, 1976; Hillson & Freedman, 1980); the results given in the present paper show that the two activities do not co-purify when a quite different purification procedure is used, namely the method for purification of bovine liver ‘thiol–protein disulphide oxidoreductase’ (Carmichael *et al.*, 1977), which involves separation of proteins on the basis of very different properties. These observations are consistent with the view that protein disulphide-isomerase and glutathione–insulin transhydrogenase activities are not alternative expressions of a single enzyme, but are distinct.

However, a satisfactory answer to the question of the relationship between the two activities depends on the complete physical resolution of these activities from one another. This has now been achieved by using a novel application of covalent chromatography to fractionate material partially purified by the conventional method for protein disulphide-isomerase (Hillson & Freedman, 1979, 1980), and the question arose whether the activities were also resolvable from material derived from the alternative purification procedure described in the present paper. We find that covalent chromatography of partially purified material [the 60–85% satn.-(NH₄)₂SO₄ fraction] obtained by using the alternative procedure of Carmichael *et al.* (1977)

also resolves the two activities. This, together with the previous results, provides final and conclusive evidence of at least two distinct enzymes catalysing thiol–disulphide oxidoreductions, one of which (protein disulphide-isomerase) is probably not responsible for insulin degradation *in vivo* since purified preparations do not show glutathione–insulin transhydrogenase activity.

The results obtained demonstrate that covalent chromatography is a very powerful technique for purification of thiol-containing proteins as a consequence of its absolute specificity for such proteins; it not only can be used to fractionate quite pure preparations, such as that used in the preceding paper (Hillson & Freedman, 1980), but it is also useful for selective purification of a small number of proteins from a relatively crude source such as the 60–85% satn.-(NH₄)₂SO₄ fraction used here. Thus considerable economies can be made both in time and materials required for a purification protocol, by using a covalent chromatographic step early in the procedure. The use of covalent chromatography for rapid purification from crude sources has been described for several proteins, including papain (Brocklehurst *et al.*, 1973), urease (Norris & Brocklehurst, 1976; Carlsson *et al.*, 1976) and procollagen type II (Angermann & Barrach, 1979).

Finally, the relationship of the so-called ‘thiol–protein disulphide oxidoreductase’, purified by Carmichael *et al.* (1977), with protein disulphide-isomerase and glutathione–insulin transhydrogenase must be considered. Purified homogeneous ‘thiol–protein disulphide oxidoreductase’ showed several activities, including reduction of the disulphide bonds of insulin (glutathione–insulin transhydrogenase-type activity) and thiol-dependent reactivation of ‘scrambled’ ribonuclease (protein disulphide-isomerase-type activity) (Morin *et al.*, 1978). Lack of quantitative data makes it impossible to draw firm conclusions on the relative importance of the various measured activities, which may reflect a broad substrate specificity, and some

of which may be fortuitous and irrelevant to the function of the enzyme *in vivo*. Purified 'thiol-protein disulphide-oxidoreductase' shows some specificity for insulin degradation, and has a mol.wt. of approx. 60000 (Morin *et al.*, 1978), which is similar to that of glutathione-insulin transhydrogenase from several sources (Varandani & Nafz, 1970; Ansorge *et al.*, 1973a). 'Thiol-protein disulphide oxidoreductase' is clearly distinct from the isomerase; the balance of evidence suggests that it corresponds to the glutathione-insulin transhydrogenase previously purified from bovine liver and the analogous enzymes purified from rat liver and bovine pancreas. However, as pointed out previously (Morin *et al.*, 1978), the structural data on these preparations show wide discrepancies. More definitive parallel structural characterization of preparations from different laboratories will be required to resolve the relationships between these activities.

References

- Angermann, K. & Barrach, H.-J. (1979) *Anal. Biochem.* **94**, 253–254
- Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Marquadt, I., Wiederanders, B. & Hanson, H. (1973a) *FEBS Lett.* **37**, 238–240
- Ansorge, S., Bohley, P., Kirschke, H., Langner, J., Wiederanders, B. & Hanson, H. (1973b) *Eur. J. Biochem.* **32**, 27–35
- Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. & Crook, E. M. (1973) *Biochem. J.* **133**, 573–584
- Carlsson, J., Olsson, I., Axen, R. & Drevin, H. (1976) *Acta Chem. Scand. Ser. B* **30**, 180–182
- Carmichael, D. F., Morin, J. E. & Dixon, J. E. (1977) *J. Biol. Chem.* **252**, 7163–7167
- Carmichael, D. F., Keefe, M., Pace, M. & Dixon, J. E. (1979) *J. Biol. Chem.* **254**, 8386–8390
- Freedman, R. B. (1979) *FEBS Lett.* **97**, 201–210
- Hawkins, H. C. & Freedman, R. B. (1976) *Biochem. J.* **159**, 385–393
- Hillson, D. A. & Freedman, R. B. (1979) *Biochem. Soc. Trans.* **7**, 573–574
- Hillson, D. A. & Freedman, R. B. (1980) *Biochem. J.* **191**, 373–388
- Ibbetson, A. L. & Freedman, R. B. (1976) *Biochem. J.* **159**, 377–384
- Morin, J. E., Carmichael, D. F. & Dixon, J. E. (1978) *Arch. Biochem. Biophys.* **189**, 354–363
- Norris, R. & Brocklehurst, K. (1976) *Biochem. J.* **159**, 245–257
- Varandani, P. T. (1974) *Biochim. Biophys. Acta* **371**, 577–581
- Varandani, P. T. (1978) in *Mechanisms of Oxidising Enzymes* (Singer, T. P. & Ondarza, R. N., eds.), pp. 29–42, Elsevier/North-Holland, New York
- Varandani, P. T. & Nafz, M. A. (1970) *Int. J. Biochem.* **1**, 313–321