Thiol–Protein Disulphide Oxidoreductases

ASSAY OF MICROSOMAL MEMBRANE-BOUND GLUTATHIONE–INSULIN TRANSHYDROGENASE AND COMPARISON WITH PROTEIN DISULPHIDE-ISOMERASE

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1. Inhibition of endogenous microsomal NADPH oxidase by CO enables membrane-bound glutathione–insulin transhydrogenase (EC 1.8.4.2) to be assayed conveniently by a linked assay involving NADPH and glutathione reductase (EC 1.6.4.2). 2. The specific activity of the enzyme in rat liver microsomal preparations is of the order of 1 nmol of oxidized glutathione formed/min per mg of membrane protein. 3. The specific activity of the enzyme is comparable in rough and smooth microsomal fractions, and the activity is not affected by treatment with EDTA and the removal of ribosomes from rough microsomal fractions. 4. Membrane-bound glutathione–insulin transhydrogenase is not affected by concentrations of deoxycholate up to 0.5%, whereas protein disulphide-isomerase (EC 5.3.4.1) is drastically inhibited. 5. On these grounds it is concluded that, in rat liver microsomal fractions, glutathione–insulin transhydrogenase and protein disulphide-isomerase activities are not both catalysed by a single enzyme species.

Enzymes catalysing oxidoreductions between thiols and disulphides have been identified from many sources (Racker, 1955; Narahara & Williams, 1959; Eriksson *et al.*, 1974; Kurane & Minoda, 1975). The earliest to be recognized were specific for low-molecular-weight thiols and disulphides (Racker, 1955) and several such activities with different specificities have been recognized in rat liver supernatants (Eriksson *et al.*, 1974).

The number, specificity and subcellular location of thiol–disulphide oxidoreductases involving protein disulphides have not been established with certainty. These enzymes are probably involved in disulphide-bond formation and scission in protein biosynthesis and degradation (Thomas, 1973; Della Corte & Parkhouse, 1973), but no unambiguous physiological role has yet been established. Gluta-thione–insulin transhydrogenase (EC 1.8.4.2, thiol-protein disulphide oxidoreductase) has been purified from several sources and studied in detail (Ansorge *et al.*, 1973; Varandani, 1973*a,b*, 1974; Chandler & Varandani, 1975*a,b*; Varandani *et al.*, 1975). Another interesting activity that should perhaps be classed as a thiol–protein disulphide oxidoreductase is the thiol-dependent protein disulphide-isomerase (EC 5.3.4.1), shown by Anfinsen, Straub and their colleagues to be capable of re-activating inactive 'wrongly' disulphide-linked ribonuclease (Goldberger *et al.*, 1963; Venetianer & Straub, 1963; Steiner *et al.*, 1965; Givol *et al.*, 1965; De Lorenzo *et al.*,

1966). For some time there has been debate as to whether this isomerase and GSH*–insulin trans-hydrogenase are distinct enzymes or different activities of a single enzyme (Tomizawa & Varandani, 1965; Katzen & Tietze, 1966; Varandani, 1973*a*, 1974).

This debate has not been resolved because it has been based mainly on comparisons of properties of purified preparations of protein disulphide-isomerase and GSH–insulin transhydrogenase from several tissues and species. The purified enzymes are clearly similar. They involve active-site thiols and act by promoting thiol/disulphide exchange (Varandani, 1973*a*). Purified ox liver GSH–insulin transhydrogenase activates reduced ribonuclease, but in general the GSH–insulin transhydrogenases do not show all the catalytic properties of ox liver protein disulphide-isomerase (Varandani, 1973*a*). Because different sources have been used, the differences in molecular weight (Varandani, 1973*a*) and in amino acid composition (Varandani, 1974) between the purified enzymes may not be conclusive.

Since both activities have been shown to be present in the microsomal fraction (Goldberger *et al.*, 1963; Varandani, 1973*a*), we have approached this problem by studying the properties of the membrane-bound enzymes. Three methods for assay

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

of GSH–insulin transhydrogenase have been described; the two that have been used for the study of the membrane-bound activity are far from direct. One involves the conversion of labelled insulin into fragments soluble in trichloroacetic acid [and hence is affected by the presence of undefined proteinases (Ansorge et al., 1973; Varandani, 1973a)]; the other follows the disappearance of insulin immunoreactivity. Neither can be regarded as following a well-defined chemical reaction. The third assay method involves detecting the formation of GSSG in the GSH–insulin transhydrogenation by coupling with NADPH and glutathione reductase (EC 1.6.4.2, NADPH–glutathione oxidoreductase) (Katzen & Tietze, 1966). This assay has been restricted to the study of purified enzyme preparations because of the turbidity of membrane preparations and interference from other NADPH-oxidizing processes in microsomal fractions (Varandani, 1973a). However, in a recent comparison of the three assay techniques (by using purified pancreatic GSH–insulin transhydrogenase), Chandler & Varandani (1975b) showed that only the NADPH-coupled assay detected the transhydrogenation between GSH and each of the three disulphide bonds of insulin.

We have therefore adapted the NADPH-coupled technique for assay of GSH–insulin transhydrogenase in rat liver microsomal preparations, and have used it to compare the properties of this activity with those of rat liver microsomal protein disulphide-isomerase. We show that, in rough microsomal fractions, the two activities respond quite differently to treatments with EDTA and with deoxycholate.

Materials and Methods

Nicotinamide, NADPH (type II), GSH, bovine serum albumin, bovine insulin and yeast glutathione reductase (type III) were supplied by Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Rotenone was purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K. SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate), as the hydrochloride salt, was a gift from Smith, Klyne and French Laboratories Ltd., Welwyn Garden City, Herts, U.K. Other reagents were of AnalaR grade, obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

The standard buffer (TKM buffer) contained 50 mM-Tris, 25 mM-KCl and 5 mM-MgCl$_2$ in double-glass-distilled water, adjusted to pH 7.5 with HCl. Sucrose solutions for membrane preparation were made up with the same concentrations of buffer salts.

Membrane preparations

Rough and smooth microsomal vesicles were pre-pared from the livers of 200 g male hooded Lister rats by centrifugation of the post-lysosomal supernatant on a discontinuous sucrose gradient (Blyth et al., 1971; Hawkins & Freedman, 1973).

Protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as standard; the volumes taken were sufficiently small that no interference from the Tris was detectable. The content of ribosomes in microsomal preparations was estimated from the RNA/protein ratio (Williams et al., 1968). RNA was determined by the method of Schmidt & Thannhauser (1945), as modified by Blobel & Potter (1968) using the extinction data of Munro & Fleck (1966). Degranulation of rough microsomal preparations was performed by using EDTA, by a modification of the method of Blyth et al. (1971). Membrane samples in 1–2 ml of 0.25 M-sucrose/TKM buffer were dialysed against 1 litre of 0.25 M-sucrose/10 mM-EDTA/50 mM-Tris/HCl, pH 7.5. 'Dialysis control' membranes were prepared by using 0.25 M-sucrose/TKM buffer as dialysis buffer. In both cases, after 24 h the dialysis residues were diluted to 5 ml with dialysis buffer. Free ribosomes were separated from EDTA-treated membranes by centrifugation over a layer of 2.0 M-sucrose in 50 mM-Tris/HCl, pH 7.5, at 105 000 g for 4 h at 4°C. 'Dialysis control' membranes were centrifuged over a layer of 2.0 M-sucrose/TKM buffer. In each case, interface material was harvested, diluted with 0.25 M-sucrose/TKM buffer, pelleted by centrifugation at 105 000 g for 1 h at 4°C, and resuspended in 0.25 M-sucrose/TKM buffer by gentle hand homogenization in an all-glass homogenizer. RNA/protein ratios for rough and smooth microsomal preparations were within the range previously reported (Hawkins & Freedman, 1973). After EDTA treatment of rough microsomal preparations the ratio was decreased to 0.05–0.06. For 'dialysis control' membranes the ratio was in the range 0.15–0.18, i.e. 83–98% of the value for the starting rough microsomal membranes. This slight decrease is presumed to be due to removal of free ribosomes present as a contaminant in the rough microsomal preparations, and also to degranulation caused by centrifugation (C. A. Blyth & B. R. Rabin, personal communication).

Enzyme assays

Studies on the rate of endogenous NADPH oxidation by microsomal preparations were performed by using 0.25 M-sucrose/TKM buffer, 0.1 mg of smooth microsomal fraction/ml and 0.1 mM-NADPH and following the oxidation of NADPH spectrophotometrically in the presence and absence of inhibitors.

Glutathione–insulin transhydrogenase activity was measured spectrophotometrically by a modification of the linked assay method of Katzen & Tietze (1966),

in which GSSG produced by the transhydrogenation reaction is reduced by NADPH and glutathione reductase. If this assay is performed by using microsomal preparations rather than partially purified glutathione–insulin transhydrogenase, i.e. by using an assay mixture comprising microsomal sample, GSH, insulin, glutathione reductase and NADPH, then a number of processes contribute to the observed oxidation of NADPH. These are (a) direct air oxidation of NADPH catalysed by the mixed-function oxidase present in microsomal preparations, (b) oxidation of NADPH by GSSG formed by the air oxidation of GSH, (c) oxidation of NADPH by GSSG formed by enzyme-catalysed glutathione–insulin transhydrogenation, and (d) oxidation of NADPH by GSSG formed by non-enzymic transhydrogenation. Direct spectrophotometric assay of microsomal transhydrogenase therefore requires the use of inhibitors to decrease the rate of process (a), and also requires correction for various background rates.

The progress of NADPH oxidation in the linked transhydrogenase assay was followed by monitoring NADPH absorbance or fluorescence. The assay mixture contained 0.125 mM-NADPH, 1.0 mM-GSH, 0.12 mM-insulin, 0.5 unit of glutathione reductase activity (1 unit catalysed the reduction of 1 μmol of GSSG/min) and up to 0.5 mg of membrane protein. Glutathione reductase was added either as a suspension in 3.6 M-$(NH_4)_2SO_4$ or after dialysis against 0.25 M-sucrose/TKM buffer; no differences were observed. Insulin was made up as a 0.6 mM solution in 50 mM-Tris/HCl, pH 7.5, containing 0.25 M-sucrose and 5 mM-EDTA. All other components were made up in 0.25 M-sucrose/TKM buffer so that the final assay mixture was in 50 mM-Tris/HCl containing 0.25 M-sucrose, 20 mM-KCl, 4 mM-$MgCl_2$, 1 mM-EDTA. In some cases, 9 mM-$(NH_4)_2SO_4$ was also present when glutathione reductase was used without prior dialysis. Buffers for all solutions were gassed with CO for 15 min before use.

Spectrophotometric assays were performed with a Perkin–Elmer 356 dual-wavelength spectrophotometer. The instrument was set at 340 nm with a 2 nm bandwidth and was operated in the split-beam mode on range 0.1 absorbance unit; 1 ml of assay mixture was examined in 1 cm-path-length cells of 1.5 ml capacity. The sample cuvette contained all five components enumerated above, but insulin was omitted from the reference cuvette. The reaction was started by the addition of NADPH to both cuvettes. The difference in NADPH oxidation between the two cuvettes was therefore a measure of glutathione–insulin transhydrogenation. The non-enzymic rate of transhydrogenation was very low; it was estimated by omitting microsomal membranes from both sample and reference cuvettes, and the enzymic GSH–insulin transhydrogenase activity could then

be deduced by difference. Alternatively the assay was carried out at two or more concentrations of microsomal membranes and the non-enzymic component was estimated by extrapolation to zero membrane concentration. Reactions were followed for 30 min. There was usually a lag phase, but rate of reaction was measured for the period from 15–30 min after mixing, during which time it was constant. Assays were normally performed in duplicate; 1 unit of enzyme activity is defined as the amount catalysing the formation of 1 μmol of GSSG/min.

Fluorimetric assays were performed on a Hitachi–Perkin–Elmer MPF3 spectrofluorimeter. The instrument was operated in the ratio mode, with excitation wavelength 350 nm, emission wavelength 460 nm and by using slits with 12 nm bandpass in both excitation and emission beams. Four cuvettes were set up: (i) contained GSH, NADPH and glutathione reductase; (ii) contained the previous components plus insulin; (iii) contained the first three components plus microsomal sample; and (iv) contained all five components. Concentrations were as for the spectrophotometric assays; 2.55 ml volumes were used. The rate of oxidation of NADPH in each of these cuvettes (i, ii, iii, iv) was measured over a period of 20–30 min. The MPF 3 spectrofluorimeter has a four-position cuvette turret, so that all four cuvettes can be monitored through a single 20–30 min period. The enzymic transhydrogenase activity is given by (iv–iii)–(ii–i).

For both spectrophotometric and spectrofluorimetric assays, cuvettes were in water-jacketed blocks maintained at 25° or at 30°C.

Protein disulphide-isomerase was assayed by the method of R. Cottrell & B. R. Rabin (R. Cottrell, personal communication); enzyme-catalysed reactivation of randomly reoxidized ribonuclease was monitored by dual-wavelength spectrophotometric assay of ribonuclease, with highly polymerized RNA as substrate. A sample of microsomal membranes was incubated with 50 μg of inactive randomly reoxidized ribonuclease in the presence of 10 μM-dithiothreitol at 30°C; the components were diluted to a total volume of 1.0 ml with 50 mM-Tris/HCl, pH 7.5, containing 0.25 M-sucrose, 25 mM-KCl and 5 mM-$MgCl_2$. Samples (10 μl) were withdrawn for ribonuclease assay at 4 min intervals for up to 30 min. The ribonuclease assay mixture contained 250 μg of RNA in 3 ml of the same buffer, and was incubated at 25°C. Ribonuclease activity was determined by following the change in E_{260} relative to the E_{280} by using the dual-wavelength mode of the Perkin–Elmer 356 spectrophotometer with a bandwidth of 2.5 nm. Ribonuclease activities were estimated from the linear change over the first 2–3 min; a plot of ribonuclease activity versus time of withdrawal gave a linear time-course for 30 min, from which protein disulphide-isomerase activity was obtained.

One unit of ribonuclease activity is defined as that producing a relative absorbance change of 1 unit/min; one unit of isomerase activity is then defined as that catalysing re-activation of ribonuclease at the rate of 1 unit/min. Correction was made for the background re-activation of randomly reoxidized ribonuclease by dithiothreitol alone. Isomerase activities of microsomal preparations, assayed by this method, were linear with membrane protein concentration up to about $200 \mu g/ml$. In general preparations were assayed at approx. $150 \mu g$ of membrane protein/ml. In our hands, optimum activity of membrane-bound isomerase was obtained by using $10 \mu M$-dithiothreitol.

Electron microscopy

Microsomal fractions were pelleted and the pellet was treated with glutaraldehyde (2.5% in 0.85M-cacodylate/HCl buffer, pH7.4) for 2h. The pellet was washed in the above buffer for 30min and then fixed in OsO_4 solution for 2h (Zetterqvist, 1965). It was then washed in buffer before being dehydrated in 60% (v/v) ethanol and left overnight. Dehydration was then continued in ethanol, and pellets were kept in ethanol for at least 2h with three changes of solvent. They were then placed in 1,2-epoxypropane for 1h, infiltrated with Mollenhaer's mixture 1 (Mollenhaer, 1964) and polymerized at 60°C for 48h. Silver sections were cut on an LKB Ultratome and stained with uranyl acetate and lead citrate. Stained sections were examined in an AEI 801A electron microscope at 60kV.

Results

Assay of membrane-bound glutathione–insulin transhydrogenase

Development of a spectrophotometric assay of GSH–insulin transhydrogenase in microsomal fractions required substantial inhibition of the
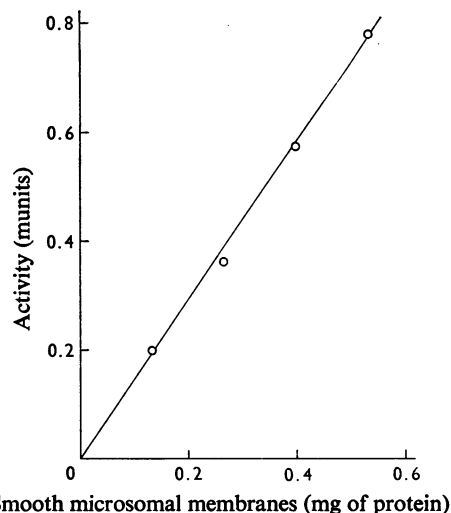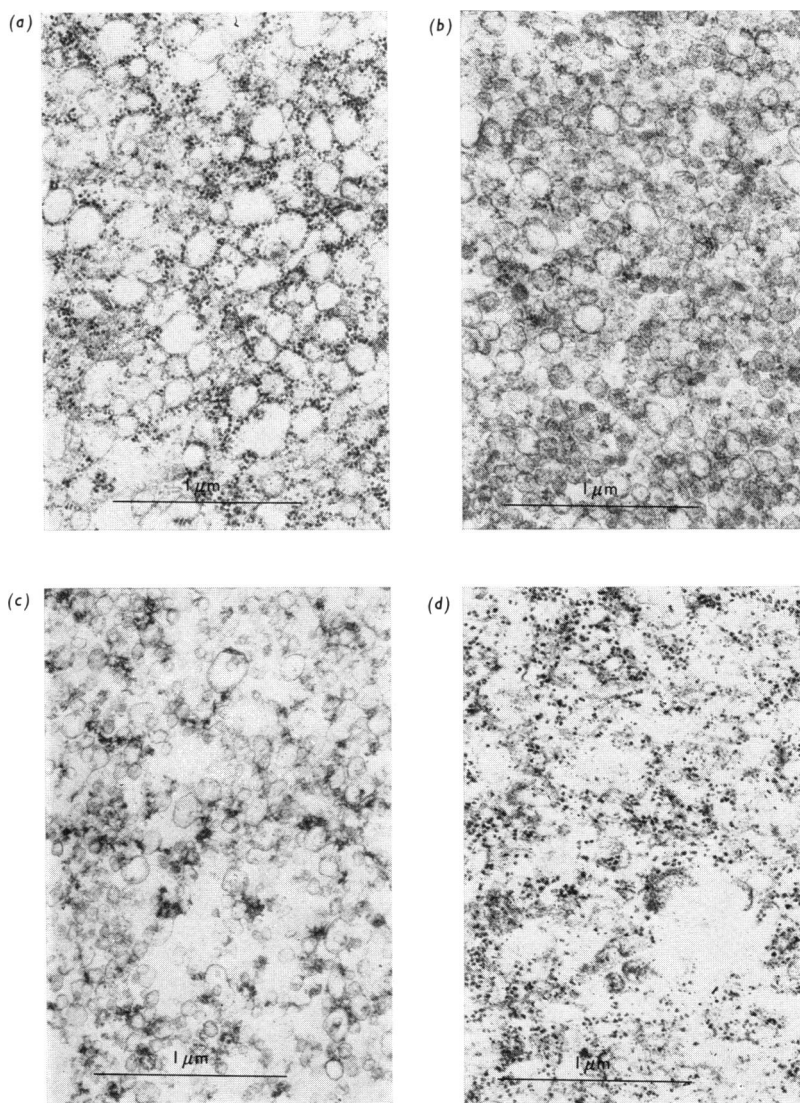

Fig. 1. *Relationship between membrane-bound glutathione–insulin transhydrogenase activity and concentration of smooth microsomal membranes*

Activities were determined as described in the Materials and Methods section.

endogenous rate of NADPH oxidation catalysed by microsomal membranes. Various compounds were tested; they included cytochrome ligands, other inhibitors of electron transport, a radical scavenger and a compound competitive with NADPH (Table 1). Significant inhibition was obtained only with CO. Treatment with CO was therefore used regularly in the preparation of solutions for transhydrogenase assay, and this greatly improved reproducibility.

The sensitivity of the Perkin–Elmer 356 spectrophotometer, operating in the split-beam mode, and its efficient optical system, which allows it to deal with high background absorbance resulting from the presence of membranes, allows membrane-bound transhydrogenase to be assayed satisfactorily. The reference cuvette contained all components except insulin, so that the air-oxidation of NADPH (catalysed by microsomal oxidase and by GSH+ glutathione reductase) is automatically corrected. The activity is linear with membrane concentration for values up to 0.5 mg of membrane protein/ml (Fig. 1). At 1mM-GSH, the K_m for insulin is $29 \mu M$ (Fig. 2). The fluorimetric assay also gave satisfactory results, but is less convenient, as each assay requires the monitoring of four cuvettes, and the rate of change in fluorescence intensity cannot be simply converted into absolute units of enzyme activity.

Table 1. *Effect of various compounds on the endogenous rate of oxidation of NADPH*

Rates were determined as described in the Materials and Methods section. The value 100% corresponds to 3.77 nmol of NADPH oxidized/min per mg.

Compound	Concentration	Relative rate
None	—	100
Compound SKF 525A	Saturated (3 mM)	100
Nicotinamide	10 mM	178
Butylated hydroxy-toluene	50 μM	76
Rotenone	10 μM	171
NaN₃	50 μM	91
CO	After gassing for 15 min	34

EXPLANATION OF PLATE I

Electron micrographs of microsomal and treated microsomal preparations

(*a*) Rough membranes; (*b*) smooth membranes; (*c*) EDTA-treated membranes; (*d*) 'dialysis control' membranes. Techniques of sample preparation are described in the Materials and Methods section.

Glutathione–insulin transhydrogenase in rough, smooth and degranulated microsomal fractions

The activities of various microsomal preparations were compared. Rough and smooth microsomal fractions are very similar in specific activity, with the rough microsomal fractions slightly the more active (Table 2). The presence of ribosomes in rough-microsomal preparations was confirmed by determination of RNA (Table 2) and by electron microscopy (Plate 1). Treatment of rough microsomal fractions with EDTA produced a very slight rise in transhydrogenase activity. EDTA treatment has been shown to degranulate rough microsomal membranes (Williams et al., 1968; Blyth et al., 1971), and this was confirmed by determination of RNA and by electron microscopy (Table 2, Plate 1). By the same criteria, dialysis in the absence of EDTA had no degranulatory effect and very little effect on enzyme activity.

Glutathione–insulin transhydrogenase and protein disulphide-isomerase

Transhydrogenase and isomerase were both assayed on the same rough and smooth microsomal preparations, and the influence of incubation with EDTA on each enzyme was studied. As shown above, transhydrogenase is of comparable activity in rough and smooth microsomal membranes and is not greatly affected by incubation with EDTA; by contrast isomerase is substantially less active in rough membranes than in smooth, but may be activated 2–3-fold by incubation with EDTA (Table 3) (Williams et al., 1968; Williams & Rabin, 1969).

Transhydrogenase and isomerase activities of rough microsomal fractions were assayed in the presence of various concentrations of deoxycholate; small volumes of 1 or 5% (w/v) sodium deoxycholate were added to membrane preparations to give final detergent concentrations up to 0.5%, and the preparations were then assayed directly. Fig. 3 shows that isomerase is inhibited significantly by very low (<0.05%) concentrations of deoxycholate,
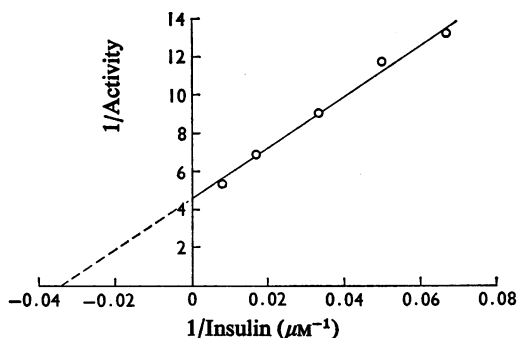
Fig. 2. *Determination of K_m for insulin of membrane-bound glutathione–insulin transhydrogenase*

Total microsomal samples (0.4mg/ml) were used. Other conditions were as described in the Materials and Methods section. One unit of enzyme activity catalysed the formation of 1 µmol of GSSG/min.

Table 3. *Comparison of glutathione–insulin transhydrogenase and protein disulphide-isomerase activities in microsomal preparations*

Activities and RNA/protein ratios were determined as described in the Materials and Methods section. Both enzymes were assayed at 30°C. Activities are expressed relative to those of rough microsomal membranes (2.20 transhydrogenase munits/mg; 3.02 isomerase munits/mg). In experiments with EDTA, microsomal membranes were incubated at 30°C in buffer containing 5mM-EDTA for 30min before enzyme assay.

Preparation	RNA/ protein	Transhydro- genase	Isomerase
Rough	0.179	100	100
Rough+EDTA	—	98	260
Smooth	0.032	92	149
Smooth+EDTA	—	—	143

Table 2. *Glutathione–insulin transhydrogenase activities and RNA/protein ratios of various microsomal preparations*

Activities and RNA/protein ratios were determined as described in the Materials and Methods section with enzyme assayed at 25°C. Activities for Expts. 1 and 2 are expressed relative to that of the rough microsomal sample (1.55 and 1.98munits/mg respectively). For Expts. 3 and 4 they are expressed relative to the corresponding 'dialysis control' sample (1.70 and 0.68munits/mg respectively). Preparations of rough, smooth, EDTA-treated and 'dialysis control' membranes are described in the Materials and Methods section.

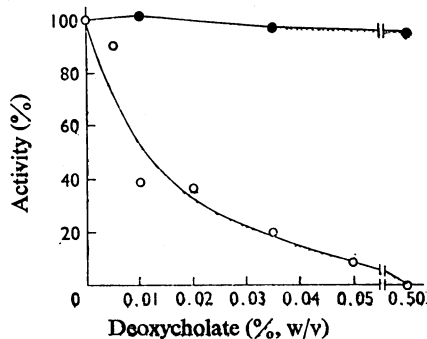| Preparation	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	Activity	RNA/protein	Activity	RNA/protein	Activity	RNA/protein	Activity	RNA/protein
Rough	100	0.189	100	0.205	—	0.213	—	0.229
Smooth	93	0.051	—	—	—	—	—	—
EDTA-treated	—	—	109	0.055	99	0.128	99	0.054
'Dialysis control'	—	—	97	0.150	100	0.180	100	0.221

Fig. 3. *Effect of deoxycholate on transhydrogenase* (●) *and isomerase* (○) *activities in rough microsomal fraction*

Activities were determined as described in the Materials and Methods section.

which have no effect on transhydrogenase. The inhibition of isomerase is not an artifact of the assay method; at the highest concentration of deoxycholate used, ribonuclease activity is still over 90% of the control.

Discussion

The inhibition by CO of endogenous microsomal NADPH oxidase activity decreases the background NADPH oxidation sufficiently for the spectrophotometric linked-assay method developed for purified GSH–insulin transhydrogenase to be applied to microsomal membrane preparations. The oxidation of NADPH by glutathione reductase and GSSG formed in the transhydrogenation reaction becomes the major contribution to the total rate of NADPH oxidation in the assay mixture; this rate can be obtained directly by using the spectrophotometer in the split-beam mode and omitting insulin from the cuvette in the control beam. The activity determined in this way is linear with microsomal concentration and gives a K_m for insulin (at 1 mM-GSH) comparable with that found by Ansorge *et al.* (1973), using a quite different method.

The spectrophotometric linked assay for GSH–insulin transhydrogenase makes it possible to express specific activity in standard units, since the corrected rate of NADPH oxidation is stoicheiometric with the rate of formation of GSSG, i.e. with the transfer of one pair of electrons in the transhydrogenation. The differences in the mode of assay makes it difficult to compare our specific activity of microsomal GSH–insulin transhydrogenase with those of other workers. Varandani (1973a) does not express activity in absolute units, but the activity measured by Ansorge *et al.* (1973)

is expressed as μg of insulin degraded/min. Microsomal preparations were found to have 9.1 such units/mg of protein. If it is assumed that the reduction of two disulphide bonds in insulin makes the molecule liable to degradation (Chandler & Varandani, 1975b), then this rate corresponds to the production of 3.2 nmol of GSSG/min per mg. In our experiments, the specific activities of GSH–insulin transhydrogenase in several microsomal preparations were within the range 0.7–2.2 nmol of GSSG formed/ min per mg. The assay conditions differ in that Ansorge's group assay the enzyme using deoxycholate-treated microsomal preparations in the presence of EDTA (Ansorge *et al.*, 1973).

The microsomal fraction contains fragments of plasma membrane, and this might be thought to be a likely location for an enzyme involved in insulin degradation (if that is the genuine physiological role of the transhydrogenase). However, the enzyme is present in both rough and smooth microsomal fractions (Tables 2 and 3), and the rough microsomal membranes are isolated by centrifugation through a sucrose solution of density 1.18 g/ml, so that they are unlikely to contain elements of plasma membrane. This finding supports the conclusion of Varandani (1973a,b) that the majority of rat liver GSH–insulin transhydrogenase is located in the endoplasmic reticulum.

Preliminary experiments on microsomal subfractionation by using zonal centrifugation in continuous sucrose gradients show that the distribution of glutathione–insulin transhydrogenase is quite different from those of plasma-membrane markers (alkaline phosphatase, 5′-nucleotidase) and corresponds closely to those for classical endoplasmic-reticulum enzymes such as NADPH–cytochrome c reductase, β-glucuronidase and arylsulphatase C (A. L. Ibbetson, R. B. Freedman, J. Tilleray & T. J. Peters, unpublished work). Thus the transhydrogenase would be classified as an enzyme of type b or e according to the nomenclature of Beaufay *et al.* (1974).

The similarity in specific activity of the enzyme in smooth and rough microsomal fractions, and the other data in Plate 1 and Table 2, demonstrate that there is no correlation between the activity of GSH–insulin transhydrogenase and the presence or absence of bound ribosomes. This contrasts with an earlier finding (Williams *et al.*, 1968; Williams & Rabin, 1969) that the activity of protein disulphide-isomerase is higher in smooth than in rough microsomal membranes, but may be activated in rough microsomal membranes by removal of ribosomes (e.g. by treatment with EDTA). The data of Table 3, in which the two activities are examined in the same membrane preparations, confirm this difference. We have demonstrated the latency of protein disulphide-isomerase in several preparations of

rough microsomal membranes, but it is a labile property frequently lost after storage. Preliminary experiments show that slow thawing of frozen suspensions is essential for retention of latency. In fact, in some cases, rough microsomal samples as prepared give protein disulphide-isomerase activities comparable with those of smooth microsomal samples, and show no latency. These results indicate that the latency of protein disulphide-isomerase may not be intimately connected with the presence of bound ribosomes; comparable RNA/protein ratios are found in rough microsomal fractions showing 'latent' or 'exposed' protein disulphide-isomerase activities. It is possible that the enzyme is intrinsically latent in rough microsomal membranes, that it can be activated by treatment with EDTA or other agents, but that it can also be activated by various storage or handling conditions, so that on occasions the enzyme may be activated during isolation of rough microsomal samples.

Activities of GSH–insulin transhydrogenase and protein disulphide-isomerase in microsomal membranes may be affected by the ease of access to active sites of the high-molecular-weight substrates insulin and randomly reoxidized ribonuclease. For this reason we investigated the effects of the detergent deoxycholate; at 0.05% deoxycholate, microsomal membranes become permeable to macromolecules, including proteins, and at 0.5% deoxycholate they are completely solubilized (Kreibich & Sabatini, 1973). The data (Fig. 3) show that deoxycholate has very little effect on the transhydrogenase even at the highest concentration, but inhibits protein disulphide-isomerase markedly. Varandani (1973a) found that Triton X-100 activates membrane-bound GSH–insulin transhydrogenase.

The differences in response to deoxycholate and those shown in Table 3 suggest that GSH–insulin transhydrogenase and protein disulphide-isomerase activities are not functions of the same enzyme. The simplest explanation is that distinct enzymes are involved (although it is conceivable that a single enzyme is operative but that one substrate, randomly reoxidized ribonuclease, makes more stringent demands on the active site than does the smaller substrate, insulin). However, the work described here does not prove that two quite distinct thiol protein-disulphide oxidoreductases are present in rat liver microsomal membranes, one entirely responsible for protein disulphide-isomerase activity and the other for GSH–insulin transhydrogenase activity. Two enzymes with different but overlapping specificities could account for the data, and perhaps more catalytic species are involved. The following paper (Hawkins & Freedman, 1976) describes an examination of this question by purification of a protein disulphide-isomerase from ox liver.

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