The latency of rat liver microsomal protein disulphide-isomerase

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1. Protein disulphide-isomerase (PDI) activity was not detectable in freshly prepared rat liver microsomes (microsomal fraction), but became detectable after treatments that damage membrane integrity, e.g. sonication, detergent treatment or freezing and thawing. Maximum activity was detectable after sonication. Identical latency was observed in microsomes prepared by gel filtration and in those prepared by high-speed centrifugation. 2. PDI activity was latent in all particulate subcellular fractions, but not latent in the high-speed supernatant. When all fractions were sonicated to expose total PDI activity, PDI was found at highest specific activity in the microsomal fraction and co-distributed with marker enzymes of the endoplasmic reticulum. 3. Washing of microsomes under various conditions that removed peripheral proteins and, in some cases, bound ribosomes did not remove significant quantities of PDI, nor did it affect the latency of PDI activity. 4. Treatment of microsomes with proteinases, under conditions where the permeability barrier of the microsomal vesicles was maintained intact, did not inactivate PDI significantly or affect its latency. 5. PDI was very readily solubilized from microsomal vesicles by low concentrations of detergents, which removed only a fraction of the total microsomal protein. 6. In all these respects, PDI resembled nucleoside diphosphatase, a marker peripheral protein of the luminal surface of the endoplasmic reticulum, and differed from NADPH:cytochrome *c* reductase, a marker integral protein exposed at the cytoplasmic surface of the membrane. 7. The data are compatible with a model in which PDI is loosely associated with the luminal surface of the endoplasmic reticulum, a location consistent with the proposed physiological role of the enzyme as catalyst of formation of native disulphide bonds in nascent and newly synthesized secretory proteins.

Protein disulphide-isomerase (PDI) (EC 5.3.4.1) catalyses thiol–disulphide interchange reactions in protein substrates. Hence, depending on the conditions, it can catalyse the formation of protein disulphide bonds from reduced proteins, the

isomerization of disulphide bonds in ‘incorrectly’ disulphide-bonded proteins, or the reduction of protein disulphide bonds by simple thiol compounds [see Freedman *et al.* (1984) for full discussion]. The enzyme was discovered in liver and pancreatic tissue over 20 years ago in the course of a search for a catalyst of the formation of native disulphide bonds in protein biosynthesis (Goldberger *et al.*, 1963; Venetianer & Straub, 1963). The wide substrate specificity of the enzyme was soon discovered, but although it was clearly plausible that the enzyme catalysed the post-translational formation of native disulphide bonds, this physiological role was not firmly established.

In the last few years, several lines of evidence have produced strong support for this hypothesis (reviewed by Freedman, 1984). Further insight into the enzyme’s physiological role could be derived

Abbreviations used: cNP, crude nuclear pellet; GIT, glutathione:insulin transhydrogenase; Man6Pase, man-nose-6-phosphatase; NDPase, nucleoside diphosphatase; PDI, protein disulphide-isomerase; (RS)ER, (rapidly sedimenting) endoplasmic reticulum; TKM, 50mM-Tris/HCl buffer, pH 7.5, containing 25mM-KCl and 5mM-MgCl₂; S-TKM, 0.25M-sucrose in the TKM buffer.

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from precise information on the enzyme's subcellular location. It is well established in a number of cases that disulphide-bond formation occurs on nascent polypeptide chains and in newly synthesized complete chains within the lumen of the ER (reviewed by Freedman, 1984). PDI was originally detected in microsomal fractions of liver and pancreas homogenates (Goldberger *et al.*, 1964), and studies with marker enzymes and isopycnic centrifugation have subsequently established that PDI is associated with the ER in sources as diverse as wheat endosperm (Roden *et al.*, 1982) and chick-embryo tendon (Brockway & Freedman, 1984). However, more detailed information is required in order to establish the enzyme's transverse and lateral distribution in the ER and to define its possible involvement in the sequence of co- and post-translational events.

The present study is an attempt to define more clearly the subcellular location of PDI in rat liver. This source was chosen because more is known about the composition and molecular organization of microsomal membranes from rat liver than those from other tissues (see, e.g., DePierre & Dallner, 1975, 1976; DePierre & Ernster, 1977). Furthermore, previous work on rat liver microsomal PDI has produced extensive, but not wholly consistent, evidence that the enzyme is latent to some extent and can be activated by solubilization or various washing treatments. No clear picture of the latency or submicrosomal location of PDI has emerged from these previous studies. Hence in the present study we have carefully re-examined the latency of rat liver microsomal PDI, re-examined the subcellular distribution of the enzyme (taking account of latency and using an assay which determines total PDI activity) and have begun a study of the submicrosomal location of the enzyme using several complementary approaches.

Experimental

Materials

Proteinases, proteinase inhibitors, assay substrates for marker enzymes, detergents and puromycin were all supplied by Sigma (London) Chemical Co. (Poole, Dorset, U.K.). All other materials were as described in Lambert & Freedman (1983a,b).

Preparation of microsomal fractions

Male Sprague-Dawley rats (150–200 g body weight, starved overnight), were killed by cervical dislocation and the livers quickly removed to ice-cold S-TKM buffer. The livers were rinsed three or four times in this buffer, blotted dry, weighed and finely minced with scissors. These steps were carried out on ice.

For preparation of microsomes (microsomal fraction) by gel filtration (Tangen *et al.*, 1973; Hawkins & Freedman, 1979) the minced liver was homogenized in 1.5 vol. of S-TKM buffer by using five up-and-down strokes of a Teflon/glass Potter homogenizer. The homogenate was centrifuged at $680g_{av}$ for 10 min and the supernatant re-centrifuged at $12000g_{av}$ for 10 min. Portions of the resultant post-lysosomal supernatant (10–20 ml $\equiv$ 10 g of liver) were loaded on to a column (2 cm $\times$ 18 cm) of Sepharose 2B equilibrated with S-TKM buffer and eluted with the same buffer at a flow rate of $2\text{ ml}\cdot\text{min}^{-1}$. Microsomal vesicles were eluted at the void volume as a thick milky suspension, well resolved from the red cytosol.

For preparation of microsomes by high-speed centrifugation, the method was essentially that of DePierre & Dallner (1976). The minced liver was homogenized in 2 vol. of S-TKM buffer by using three up-and-down strokes of the Potter homogenizer. The homogenate was centrifuged at $680g_{av}$ for 10 min, and the pellet re-homogenized in an equal volume of buffer and centrifuged again at $680g_{av}$ for 10 min. The re-extracted pellet (cNP) was discarded and the supernatant combined with that from the initial centrifugation. This post-nuclear supernatant was centrifuged at $12000g_{av}$ for 10 min and the lysosomal pellet discarded. The resultant supernatant was centrifuged at $100000g_{av}$ for 60 min. The final supernatant (cytosol fraction) was discarded and the pellet (microsomes) was suspended in 20 ml of 0.15 M-Tris/HCl, pH 8.0, by using a glass homogenizer, and then re-centrifuged at $100000g_{av}$ for 60 min. The pellet (washed microsomes) was taken up in the same buffer at about 5–10 mg of protein/ml, and the supernatant (Tris wash) was discarded.

Subcellular fractionation

The basic method for subcellular fractionation paralleled that for preparation of microsomes by high-speed centrifugation, except that the discarded fractions (cNP, lysosomal pellet, cytosolic fraction and Tris wash) were retained and resuspended or diluted where appropriate with S-TKM.

Further fractionations of the cNP were as follows. The pellet was resuspended in S-TKM and TKM added to give a final sucrose concentration of 80 mM. The suspension was centrifuged on a discontinuous sucrose-density gradient and resolved into a nuclear pellet and an RSER fraction containing mitochondria and sheets of ER by using the method of Shore & Tata (1977). Alternatively the cNP was taken up in a sucrose/Hepes buffer and processed to give a purified mitochondrial fraction by the method of Pedersen *et al.* (1978).

Sonication

Samples to be sonicated for assay of 'total' PDI activity were diluted in TKM buffer to approx. 3–5 mg of protein/ml. Portions (6 ml) were sonicated in an ice bath with an MSE 150W probe sonicator (1.7 cm diameter probe) at an amplitude of 9–11 μ m peak-to-peak for 3 $\times$ 15 s bursts, with 15 s intervals.

Assay of PDI and definition of percentage latency

PDI activity was assayed as previously described (Lambert & Freedman, 1983b) with TKM as the buffer for the incubation and the ribonuclease assay, unless otherwise mentioned. The lower limit of detection of PDI activity was as discussed by Lambert & Freedman (1983b). The latency of PDI (or of other enzymes) was defined as:

$$[(A_s - A_0)/A_s] \times 100$$

where A_s is the activity after sonication and A_0 is the activity of the untreated sample under iso-osmotic conditions.

Assays of markers

NDPase was assayed with UDP as substrate by using the stopped-assay method of Ishibashi *et al.* (1978), and the phosphate released was determined by the method of Baginski *et al.* (1974). Man6Pase activity was assayed by the method of Baginski *et al.* (1974), but 2 mM-mannose 6-phosphate rather than glucose 6-phosphate was used (Nilsson *et al.*, 1978). Lactate dehydrogenase was assayed spectrophotometrically as described by Bergmeyer & Bernt (1974). NADPH:cytochrome *c* reductase was assayed spectrophotometrically by monitoring the reduction of cytochrome *c* at 550 nm as described by Phillips & Langdon (1962), the difference absorption coefficient for cytochrome *c* given by Hatefi & Stiggall (1978) being used. Glutamate dehydrogenase activity was assayed in the direction 2-oxoglutarate $\rightarrow$ glutamate by the method of Schmidt (1974). Acid phosphatase was assayed, with *p*-nitrophenol phosphate as substrate, by using the stopped-assay method of Walter & Schutt (1974). Succinate dehydrogenase was assayed with 2,6-dichlorophenol-indophenol as oxidant and phenazine methosulphate as mediator as described by Ackrell *et al.* (1978). Phenazine methosulphate solutions were stored on ice in covered vials and used within 2 h of preparation. RNA was determined as previously described (Hawkins & Freedman, 1979). In all cases appropriate sample- and reagent-blank assays were performed and corrected for.

Degranulation

The method used was that of Hawkins & Freedman (1979). Microsomal pellets were prepared by the high-speed-centrifugation method

above, except that the buffer throughout was 250 mM-sucrose/50 mM-Tris/HCl, the KCl and MgCl₂ being omitted. The pellets were then resuspended in 50 mM-Tris/HCl buffer, pH 7.5, containing 250 mM-sucrose, 500 mM-KCl and either 20 mM-EDTA or 1 mM-puromycin, incubated for 30 min on ice and fractionated by gel filtration on Sepharose 2B columns (2 cm $\times$ 18 cm) eluted with sucrose/Tris/HCl buffer.

Proteinase treatments

Gel-filtered microsomes (25–35 mg of protein) were incubated with proteinases (50 or 100 μ g/mg of microsomal protein) in a total volume of 4 ml of S-TKM buffer for 15 or 30 min at 32°C. In the case of trypsin, digestion was stopped by addition of soya-bean trypsin inhibitor (5 μ g/ μ g of trypsin) and dilution to 8 ml with S-TKM buffer. Digested microsomes were collected by centrifugation at 100 000 g_{av} for 60 min, washed in S-TKM buffer, re-centrifuged and finally resuspended in 6 ml of S-TKM. In some cases the washing buffer contained 500 mM-KCl and the sample was incubated in this for 20 min on ice before re-centrifugation. At the end of digestions with chymotrypsin or subtilisin, samples were diluted with 4 ml of S-TKM buffer and centrifuged at 100 000 g_{av} for 1 h. Microsomes were resuspended in 3 ml of 150 mM-Tris/HCl, pH 7.5, and proteinase inhibitors added. For incubations with 50 and 100 μ g of subtilisin/mg of microsomal protein, 0.02 and 0.04 ml of phenylmethanesulphonyl fluoride (300 mM in ethanol) were added respectively; for incubations with 50 and 100 μ g of chymotrypsin/mg, 0.02 and 0.04 ml of *N*-tosyl-L-phenylalanylchloromethane ('TPCK') (150 mM in methanol) were added respectively. These samples were incubated at 25°C for 20 min, 3 ml of 1 M-KCl was added, and, after a further 15 min on ice, the samples were centrifuged and resuspended in 6 ml of S-TKM. In all cases four or five samples were treated and assayed together, one of which was a control sample from which proteinase was omitted.

Results and discussion

Latency of microsomal PDI

It was noted that rat liver microsomes, prepared by the ultracentrifugation method described above and assayed immediately in TKM buffer containing 0.25 M-sucrose, had no detectable protein disulphide-isomerase activity, whereas the same microsomes when frozen and thawed and then assayed under the same conditions, or when assayed directly in TKM buffer alone, had readily detectable PDI activity. The results in Table 1 indicate that the effects of freezing and thawing and of assay under hypo-osmotic conditions are

Table 1. *Effects of membrane-disruptive treatments on microsomal PDI activity*

Microsomes were prepared and maintained in S-TKM buffer; they were assayed for PDI activity in either S-TKM (iso-osmotic assay buffer) or TKM (hypo-osmotic assay buffer). In the freeze-thaw experiments, the microsomal suspension was at 6–8 mg of protein/ml. For detergent treatment, 1 ml of microsomal suspension was incubated with 1 ml of 1% (w/v) Triton X-100 for 30 min on ice; portions (0.05 ml) were taken for assay in a final volume of 1 ml, giving a concentration of 0.025% (w/v) Triton in the assay incubation mixture. Studies with homogeneous bovine liver PDI indicate that Triton X-100 at this concentration in the assay incubation inhibits the enzyme by 40–50%. Sonication was carried out as described in the text. Results are means $\pm$ S.D. for the number of determinations given in parentheses. Microsomes prepared either by ultracentrifugation or by gel filtration were used, since they were not significantly different (see Table 2).

Microsome sample	Assay buffer ...	PDI activity (units/g of protein)	
		Iso-osmotic	Hypo-osmotic
Fresh		0 (8)	2.5 $\pm$ 0.3 (8)
Frozen 48 h, -20°C		2.7 $\pm$ 0.2 (6)	5.2 $\pm$ 0.9 (6)
Detergent-treated (0.5% Triton X-100)		9.4 $\pm$ 1.2 (6)	9.1 $\pm$ 1.1 (6)
Sonicated		16.0 $\pm$ 1.3 (8)	17.1 $\pm$ 1.1 (8)

Table 2. *Comparison of microsomal fractions prepared by ultracentrifugation and gel filtration*

Microsomes were prepared by the methods described in the text. Enzyme activities were assayed before (–) and after (+) sonication. Units of PDI are as defined by Lambert & Freedman (1983b); a unit of the phosphatase activity is the amount releasing 1 μmol of phosphate/min. The results presented are means $\pm$ S.D. for six experiments. Microsomes prepared by the two methods also had closely similar levels of cytochrome *P*-450 (0.63–0.64 $\mu\text{mol/g}$ of protein). The activities of these markers, and the levels of markers of contaminating organelles (succinate dehydrogenase, acid phosphatase, lactate dehydrogenase), were comparable with those obtained by other workers (Tangen *et al.* 1973; Amar-Costesec *et al.*, 1974; Nilsson *et al.*, 1978; Ohba *et al.*, 1981).

Enzyme	Method ... Sonication ...	Enzyme activity (units/g of protein)			
		Ultracentrifugation		Gel filtration	
		–	+	–	+
PDI		0	18 $\pm$ 2	0	16 $\pm$ 2
NDPase		87 $\pm$ 5	1160 $\pm$ 220	85 $\pm$ 6	985 $\pm$ 125
Man6Pase		16 $\pm$ 3	200 $\pm$ 8	14 $\pm$ 2	175 $\pm$ 10

additive, and that higher levels of PDI activity are recorded after disruption of microsomal membranes by detergents or by sonication. In the studies on detergent-treated microsomes, the assays are in the presence of low concentrations of detergents carried through with the sample, and the values observed reflect the direct inhibitory effect of detergent on soluble PDI, in addition to the activating effect of solubilization [see Freedman *et al.* (1978) for full discussion]. By contrast, the activities observed after sonication are reproducible, are the highest recorded after any treatment and show no significant variation with more extended sonication; hence the activity observed after sonication can be taken as a measure of the total PDI present.

In view of previous observations suggesting that PDI is solubilized simply by resuspension of a microsomal pellet (see, e.g., Drazic & Cottrell, 1977), we examined microsomal fractions prepared by gel-filtration, in parallel with microsome

fractions prepared by ultracentrifugation. The two preparations (see the Experimental section) were entirely comparable in the following respects: (i) the complete absence of PDI activity before sonication; (ii) the specific activity of PDI observed after sonication; (iii) the latency of Man6Pase and NDPase activities (Table 2). They were also comparable in their levels of RNA, cytochrome *P*-450 and NADPH: cytochrome *c* reductase and in their low levels of contamination by markers of other organelles. Hence microsomes for study of PDI latency may be isolated either by gel filtration or by centrifugation methods. However, repeated ultracentrifugation and resuspension (three or more treatments at 105 000g) led to some solubilization of PDI, as previously observed, and to loss of latency.

Subcellular location of total PDI activity

The findings above show that microsomal PDI is entirely latent in the absence of treatments that

damage membrane integrity. Since this latency has not been fully appreciated in previous studies of the enzyme's subcellular location, the question was re-examined. A rat liver homogenate was subfractionated by conventional differential centrifugation, and the activity in each fraction was determined before and after sonication. The results are shown in Fig. 1. The microsomal fraction had the highest specific activity of PDI, when assayed after sonication, and the activity was >95% latent; PDI also showed latency in the other particulate fractions, but it is noticeable that PDI activity in the high-speed supernatant fraction was *not* latent.

With the protocol used here, the cNP contained nuclei, mitochondria and RSER associated with mitochondria (Shore & Tata, 1977; Meier *et al.*, 1981). An enriched nuclear fraction and, in another experiment, a purified mitochondrial

fraction were prepared from this cNP and, after sonication, had specific PDI activities of 4units and 2units/g of protein respectively, below those of the homogenate and of the cNP. These findings suggest that the PDI present in cNP is associated with sheets and fragments of ER, rather than with nuclei or mitochondria.

The implication from all these studies, that PDI is located in the ER, was confirmed by marker-enzyme studies. Table 3 shows that the distribution of PDI corresponds closely to that of NDPase and (less closely) to that of NADPH:cytochrome *c* reductase, both markers of the ER, and is quite distinct from the distribution of markers of other organelles. Recoveries were similar for all the markers, and markers for mitochondria, lysosomes and cytosol were found in the expected fractions. These data are the first full characterization of the subcellular location of PDI in rat liver using a wide range of marker enzymes and taking account of the latency of PDI. They confirm that the rat liver enzyme is located in the ER, as has been suggested by less comprehensive studies on the enzyme from wheat endosperm (Roden *et al.*, 1982) and chick-embryo tendon (Brockway & Freedman, 1984).

The abundance of PDI in microsomal fractions can be calculated from its specific activity. Under the assay conditions used in the present study, the specific activity of homogeneous rat liver PDI is calculated to be 770units/g (Lambert & Freedman, 1983*b*; Mills *et al.*, 1983). Since the specific activity of total PDI in microsomal fractions is approx. 17units/g, PDI forms ($17/770 = 0.022 =$) 2.2% of the total microsomal protein. Ohba *et al.* (1981) have demonstrated by immunoassay that PDI comprises 1.5% of total rat liver microsomal protein and that it is present at equal levels in both rough and smooth microsomes.

Effects of washing procedures on microsomal PDI

Since the assay of PDI involves a macromolecular substrate, 'scrambled' ribonuclease

Fig. 1. Subcellular distribution of PDI activity Subcellular fractions were prepared from a rat liver homogenate as described in the text. The five fractions generated (a, cNP; b, lysosomal pellet; c, washed microsomal pellet; d, cytosol; e, Tris wash) were assayed for PDI activity before and after sonication; the shaded regions represent the value obtained before sonication. The broken line represents the specific activity of the crude homogenate after sonication.

Table 3. Subcellular fractionation of rat liver

Samples were sonicated before analysis. Results are means $\pm$ s.d. for three subfractionations. Recovery of protein was 90%; recoveries of the enzyme markers were in the range 80–89%. Further enzyme abbreviations: GDH, glutamate dehydrogenase; SDH, succinate dehydrogenase; NCCR, NADPH:cytochrome *c* reductase; LDH, lactate dehydrogenase; AP, acid phosphatase.

Fraction	Proportion (% of homogenate value)							
	Protein	GDH	SDH	AP	NDPase	PDI	NCCR	LDH
cNP	44 $\pm$ 5	75 $\pm$ 5	69 $\pm$ 10	21 $\pm$ 3	31 $\pm$ 2	37 $\pm$ 2	24 $\pm$ 2	13 $\pm$ 3
Lysosomal pellet	9 $\pm$ 1	7 $\pm$ 1	7 $\pm$ 1	34 $\pm$ 3	10 $\pm$ 1	8 $\pm$ 1	11 $\pm$ 1	4 $\pm$ 1
Microsomes	10 $\pm$ 1	1	0	10 $\pm$ 2	31 $\pm$ 3	26 $\pm$ 3	45 $\pm$ 5	1
Cytosol	25 $\pm$ 3	5 $\pm$ 1	4 $\pm$ 1	15 $\pm$ 2	9 $\pm$ 3	10 $\pm$ 1	3 $\pm$ 1	65 $\pm$ 11
Tris wash	2	<1	0	2 $\pm$ 1	1	2	<1	6 $\pm$ 1

($M_r \sim 13000$), the enzyme's latency in undamaged microsomal vesicles could reflect the limited accessibility of the enzyme active site to this substrate. The structural basis of this effect was studied by using various washing treatments, proteinases and detergents.

Microsomes prepared by ultracentrifugation without the standard washing step were immediately resuspended in various aqueous buffers known to remove peripheral or 'artefactually' adsorbed proteins from the external (cytosolic) surface. The residual microsomes and solubilized proteins were separated by ultracentrifugation, and were then assayed for PDI activity before and after sonication. Table 4 shows the percentages of total protein and of total PDI solubilized. Although up to 25% of microsomal protein was solubilized by these procedures, no more than 5% of total PDI was removed. The recovery of PDI in the washed microsomes was always more than 90%, and the latency of PDI in these washed microsomes was always more than 95%. Hence PDI is not a peripheral protein at the cytosolic surface of the vesicles, nor is it masked by such a protein.

Treatments with EDTA and with puromycin are known to displace membrane-bound ribosomes. To confirm that such 'degranulation' had no effect on microsomal PDI activity, or on its latency, microsomes were incubated with 500 mM-KCl in the presence of either 1 mM-puromycin or 20 mM-EDTA, and the residual microsomal vesicles were resolved from displaced proteins and ribosomes by gel filtration on Sepharose 2B (see the Experimental section). Both procedures decreased the RNA/protein ratio of the vesicles from 0.144 to 0.066, i.e. they displaced more than 50% of microsomal RNA, but in both cases the microsomal PDI was still entirely latent (>95%) and the specific activity observed after sonication was 18 units/g of protein.

Effects of proteinases on microsomal PDI

Microsomes prepared by gel filtration were incubated with trypsin under various conditions; proteolysis was stopped by the addition of soya-bean trypsin inhibitor, and the treated microsomes were recovered by centrifugation. Control experiments established that these procedures completely inhibited the proteinase and removed it from the microsomes before subsequent assays. In preliminary studies it was found that, if the proteinase was inactivated and removed from microsomes simply by cooling to 4°C and centrifugation to recover microsomes, some proteinase was carried over, leading to irreproducible results and the partial degradation of known luminal-surface markers.

The results in Table 5(a) show that trypsin treatment under controlled conditions removed up to 30% of microsomal protein and completely inactivated NADPH:cytochrome *c* reductase, an integral microsomal enzyme known to be exposed at the cytosolic surface. The latency and total activity of nucleoside diphosphatase (a lumenally located protein) were not significantly affected by trypsinolysis, indicating that the integrity of the microsomes had not been perturbed. Similarly, the latency of PDI remained at >90% after all the trypsin treatments, and even the most severe treatment resulted in only 25% loss of total PDI activity. Trypsin rapidly inactivates purified PDI in solution (results not shown), and both NDPase and PDI were significantly inactivated when sonicated microsomes were incubated with trypsin under the same conditions; these enzymes are therefore susceptible to trypsin digestion when the proteinase had unrestricted access. Incorporating a wash with 500 mM-KCl after trypsinolysis (Meyer & Dobberstein, 1980) increased the total amount of protein solubilized, but had insignificant effects on the recoveries of microsomal enzymes.

To test whether the resistance of microsomal

Table 4. *Effects of washing procedures on microsomal PDI*

Microsomes (25–30 mg) prepared by 100000g centrifugation of post-lysosomal supernatants were resuspended in one of the above buffers (6 ml), left on ice for 20–30 min and re-pelleted at 100000g. The supernatants were assayed for protein and PDI activity. The pellets were taken up in S-TKM, and PDI activity was measured before and after sonication. The results are means $\pm$ S.D. for three separate experiments. Recoveries of total protein and PDI activity were always 90–100%. Latency was more than 95% in all cases.

Washing procedure	Protein removed (%)	PDI removed (%)
S-TKM	4.8 $\pm$ 0.7	
150 mM-Tris/HCl, pH 8.0	21.3 $\pm$ 2.0	5.9 $\pm$ 0.2
50 mM-Tris/HCl (pH 7.5)/500 mM-KCl	24.5 $\pm$ 1.5	5.1 $\pm$ 0.1
50 mM-Tris/HCl (pH 7.5)/500 mM-KCl/1 mM-puromycin	22.0 $\pm$ 1.7	4.9 $\pm$ 0.7
50 mM-Tris/HCl (pH 7.5)/250 mM-sucrose/5 mM-EDTA	5.3 $\pm$ 0.6	

Table 5. *PDI and marker-enzyme activities in microsomes treated with proteinases*

Microsomes were incubated with proteinases as described in the text. After recovery and resuspension of the treated microsomes, protein and enzyme determinations were carried out before and after sonication. Results are means for three experiments. (a) NDPase and PDI activities were over 90% latent in all cases. Control activities after sonication were: PDI, 15.8units/g; NDPase, 1250units/g NCCR (NADPH:cytochrome *c* reductase), 27units/g. N.D., not determined. (b) NDPase, Man6Pase and PDI activities were over 85% latent in all cases. Control activities after sonication were: PDI, 18.1units/g; NDPase, 970units/g; Man6Pase, 210units/g; NCCR (NADPH:cytochrome *c* reductase), 35units/g.

		Total amount or activity (%)					
(a)	Treatment	Protein	PDI	NDPase	NCCR		
	Control	100	100	100	100		
	Trypsin						
	50 µg/mg for 15 min	87	88	95	45		
	50 µg/mg for 30 min	80	86	97	18		
	100 µg/mg for 30 min	73	75	91	8		
	Trypsin (50 µg/mg) for 30 min with a 500mM-KCl wash	66	83	92	15		
	Trypsin (50 µg/mg of sonicated microsomes) for 30 min	N.D.	48	35	N.D.		
		Total amount or activity (%)					
(b)	Treatment	Concn. (µg/mg)	Protein	PDI	NDPase	M6Pase	NCCR
			100	100	100	100	100
	Chymotrypsin	50	62	94	90	94	30
		100	58	80	92	84	5
	Subtilisin	50	58	91	100	95	26
		100	56	78	92	82	4

PDI was a general phenomenon or determined by the specificity of trypsin as a proteinase, microsomes were also digested with chymotrypsin or subtilisin. Careful controls were carried out to ensure that the procedure stopped proteolysis after a defined time and removed the proteinase before the microsomal enzymes were assayed (see the Experimental section). The data in Table 5(b) show that proteinase treatments that remove over 40% of microsomal protein and completely inactivate NADPH:cytochrome *c* reductase have very little effect on the activities of PDI and of the markers NDPase and Man6Pase. After chymotrypsin or subtilisin treatments all three enzymes were still more than 85% latent.

Susceptibility to proteinases has been widely used as a probe of the tranverse disposition of membrane proteins (see, e.g., Etemadi, 1980) and specific enzymes of rat liver microsomes have been ascribed a tranverse location on the basis of their sensitivity (e.g. NADPH:cytochrome *c* reductase) or insensitivity (e.g. NDPase) to proteinase treatments (DePierre & Dallner, 1975; DePierre & Ernster, 1977). To interpret such studies it is vital that the proteolysis proceeds under controlled conditions in which the permeability barrier of the membrane is retained and that no proteinase survives to act later in the work-up procedure when the membranes may be solubilized before assay.

The above results from studies under well-controlled conditions show that microsomal PDI was resistant to three proteinases differing in specificity, all of which inactivated the cytosolic surface marker NADPH:cytochrome *c* reductase by more than 90%.

Not only was PDI activity retained in proteinase-treated microsomes, but its latency was retained. Hence the active site of the enzyme is not at the cytosolic surface and is not masked by another protein component which can be removed from this surface by controlled proteolysis. Specifically, PDI is not masked by the protein component postulated to interact with the signal receptor particle, a complex of RNA and proteins which interacts with the *N*-termini of nascent secretory polypeptides and is proposed to mediate their insertion in the ER membrane. The membrane protein interacting with this particle ('docking protein') is known to be cleaved by trypsin, and a 60kDa tryptic peptide is then removed by washing with high salt (Meyer & Dobberstein, 1980; Gilmore *et al.*, 1982*a,b*). This treatment was found to have no effect on the latency of PDI (Table 5*a*).

Solubilization of microsomal PDI by detergents

The findings on the insensitivity of microsomal PDI to proteinases indicate that the active site of the enzyme is located inside the permeability

Fig. 2. Solubilization of microsomal enzymes by detergents. Gel-filtered microsomes (30–35 mg of protein) were incubated with either sodium deoxycholate or Triton X-100 at the final concentrations noted, in a total volume of 5 ml of S-TKM buffer, for 30 min on ice. Microsomes were recovered by ultracentrifugation ($100000g_{av.}$, 60 min) and re-centrifuged in S-TKM. The final pellet was resuspended in 6 ml of S-TKM for assay; enzyme and protein assays were performed after sonication, either on the day of microsomal preparation, or after overnight storage at -20°C . The ordinate represents the amounts of protein and enzymes retained in the microsomal

barrier of the microsomal vesicles. Further evidence on the enzyme's interaction with the vesicles was obtained from studies with detergents.

Gel-filtered microsomes were incubated with low concentrations of Triton X-100 or sodium deoxycholate and the residual microsomes were recovered by centrifugation. The pellets were resuspended and were analysed for protein and enzyme activities after sonication. Fig. 2 shows that microsomal protein was increasingly solubilized with increasing detergent concentration, 40% of the total protein being solubilized by 0.2% (w/v) Triton and 0.1% (w/v) deoxycholate respectively. The integral membrane enzyme Man6Pase was only solubilized to a small extent; comparable results were obtained with NADPH:cytochrome *c* reductase (results not shown). However, both PDI and NDPase were very readily solubilized by both detergents, 0.2% (w/v) Triton and 0.1% (w/v) deoxycholate causing 90% solubilization of both enzymes.

Kreibich *et al.* (1973) showed that very low levels of detergents increased the permeability of microsomal vesicles to proteins, without totally disrupting the vesicles, and selectively released a defined set of proteins. This set contains several enzymes, including NDPase (see Nilsson & Dallner, 1975), but it is not clear whether these enzymes exist free in the lumen or are loosely associated with the luminal surface of the vesicles. [This distinction cannot be made confidently even for newly synthesized secretory proteins, e.g., proalbumin (Vlasuk *et al.*, 1980)]. The results in Fig. 2 show that PDI, like NDPase, is selectively released by non-disruptive detergent concentrations, and suggest that it is associated with the luminal surface of ER membranes.

Comparison with previous studies on PDI

Previous work on liver microsomal PDI has shown that various treatments activate the enzyme to some extent. The treatments include lipid extraction with acetone followed by buffer solubilization from the acetone-dried power (Givol *et al.*, 1964; De Lorenzo *et al.*, 1966; Hawkins & Freedman, 1976; Ohba *et al.*, 1981), solubilization by detergents (Ohba *et al.*, 1977, 1981; Freedman *et al.*, 1978), centrifugation and resuspension (Drazic & Cottrell, 1977; Ohba *et al.*, 1977) and treatments leading to displacement of ribosomes (Williams *et al.*, 1968; Dani *et al.*, 1976). In all these studies some PDI activity was detected in

pellet as a percentage of that retained in microsomes treated comparably but in the absence of detergent. □, Protein; ●, PDI; ○, NDPase; ■, Man6Pase.

apparently untreated microsomes, and previously quoted values for PDI activity in untreated rat and bovine liver microsomes range up to 4 units/g of protein. The procedures in these previous studies varied widely, but all involved extensive ultracentrifugation and washing and/or storage as frozen suspensions. The findings in the present work (Table 1) make it clear that PDI is completely latent in fresh untreated microsomal fractions, and that the low and variable activities previously found reflect various extents of damage to vesicle integrity in those previous preparations. The present results also establish that latent PDI activity is exposed to some extent by any treatment tested which can damage membranes. Latency of PDI is not retained during assay under hypotonic conditions (Table 1) or even after 24 h storage at -20° in the presence of 20% (v/v) glycerol (results not shown). Hence the latency of PDI is a very sensitive and labile property, as previously noted (Ibbetson & Freedman, 1976).

PDI was latent in all particulate subcellular fractions. It was also detectable in the soluble fractions, where it was not latent; if sonication had not been used to determine total PDI activity, this minor fraction (presumably released by disruption of the ER during homogenization and differential centrifugation) would have led to the enzyme's being ascribed a cytosolic location. For example, in a previous study, Ohba *et al.* (1981) found that, of the PDI activity in a postnuclear supernatant, 60% appeared in the cytosolic fraction after subfractionation and 40% in the microsomal fraction. However, when the enzyme was detected immunologically, rather than by enzyme activity, 60% was found in the microsomal fraction and only 5% in the cytosolic fraction (in agreement with our data based on assay of total PDI activity). These results indicate that their enzyme assay was not detecting total PDI activity and was underestimating PDI in the particulate fractions in which it was latent.

Similarly, in previous work (Drazic & Cottrell, 1977; Ohba *et al.*, 1977, 1981) it was claimed that substantial amounts of PDI activity could be washed from microsomal membranes by high-salt or other aqueous buffers. However two aspects of these results were puzzling. Firstly, in the work of Drazic & Cottrell, the total activity after washing (released + residual) was considerably greater than that reported as initially present in the microsomes. Secondly, Ohba *et al.* (1981) found that only a small fraction of PDI was released on washing, when determined by immunoassay, whereas 50% was released when determined by enzyme activity. The inconsistencies in these results, and the discrepancies between these findings and ours (Table 4), can be explained by the fact that in the earlier studies no steps were taken to overcome

latency, and so the enzymic assays of PDI in the initial microsomes were considerable underestimates. Washing solubilized a small fraction of PDI which was then not latent and therefore appeared as a very significant proportion of the total. By taking full account of latency and employing an assay procedure which determines total PDI activity, we have obtained fully consistent results showing that PDI is not readily solubilized by aqueous washes, and that its latency is maintained.

Comparison with data on GIT

PDI can also catalyse the reduction of insulin disulphide bonds by glutathione (Varandani, 1978; Bjelland *et al.*, 1983; Lambert & Freedman, 1983a,b). This alternative activity (GIT) has been extensively studied.

Varandani (1973) established that the bulk of GIT activity in rat liver was associated with the microsomal fraction, and a recent careful study by isopycnic centrifugation (Chowdhary *et al.*, 1983) has shown that GIT activity co-distributes with markers of the ER and is not associated with plasma-membrane fragments or with other organelles. It was also found that GIT activity was latent in intact microsomes and could be activated by a number of membrane-disruptive treatments including vigorous Polytron homogenization, freeze-thawing, sonication, detergent-solubilization and treatment with phospholipases (Varandani, 1973; Hern & Varandani, 1983a).

The observations by Hern & Varandani (1983a), in particular, correspond quite closely to those reported here, in that GIT was found to be latent in microsomal fractions, to be activated by freeze-thawing, to be solubilized by low concentrations of detergents and to be resistant to proteinase attack in intact microsomes but sensitive in permeabilized microsomes. As a result, these workers concluded that the enzyme was similar in orientation to NDPase and was weakly bound to the cisternal surface of microsomal vesicles, i.e. to the luminal surface of the ER. The major discrepancy between these findings and ours is that Hern & Varandani (1983a) detected GIT activity in supposedly intact microsomes and concluded that approx. 15% of the total GIT activity was non-latent. Attempts have been made to ascribe physiological significance to this 'non-latent' GIT activity (Hern & Varandani, 1983b). However, the preparation of microsomes used in their studies involved a potentially damaging centrifugation at 160000g, and microsomal membranes were stored frozen and thawed before use. No control studies were performed with known luminal-enzyme markers to assess the integrity of the microsomal vesicles, and it seems likely that some membrane damage was induced by this sequence of treatments. Hence the

proposed 15% non-latent GIT activity is probably an artefact.

Conclusions

This work has established more soundly than before that, in rat liver, PDI is located in the ER. A conclusion on the location of PDI within this membrane requires the interpretation and integration of data from several independent approaches. Each technique has its limitations and uncertainties, and is inadequate in isolation. Nevertheless, the present data indicate that PDI is latent in intact microsomal vesicles, that it is not affected by various aqueous washes or by proteinase treatments of microsomes, but that it is readily solubilized by detergents and activated by a variety of membrane-damaging treatments. A self-consistent interpretation of these findings is possible as follows.

The enzyme active site is inaccessible to macromolecules, namely its protein substrate and a range of proteinases. This inaccessibility was also observed by Ohba *et al.* (1981), who found that microsomal PDI bound significantly less anti-PDI antibodies than did soluble PDI, and that the glutamine residues of the enzyme in microsomes were not labelled by transglutaminase. This suggests that the bulk of the enzyme is located inside the membrane-permeability barrier. The resistance to solubilization by high salt and other washing treatments, and the contrasting ready solubilization by detergents, suggest that the enzyme is located at the luminal surface, or within the luminal content of the vesicles. This conclusion is therefore consistent with many lines of evidence, and it is also consistent with the enzyme's proposed physiological role; an enzyme catalysing formation of native disulphide bonds in nascent and newly synthesized proteins would be expected to act within the lumen of the ER.

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