Dog pancreatic microsomal-membrane polypeptides analysed by two-dimensional gel electrophoresis

Mustak A. KADERBHAI and Brian M. AUSTEN

Department of Surgery, St. George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

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The two-dimensional polyacrylamide-gel electrophoresis technique of O'Farrell [(1975) *J. Biol. Chem.* **250**, 4007–4021] was applied to resolve and analyse the polypeptide composition of dog pancreatic rough microsomal membranes, which were shown to be active in co-translational processing of preprolactin synthesized from pituitary mRNA in a translation system *in vitro*. About 100 polypeptides are resolved. Treatment of rough microsomal membranes with EDTA and high KCl concentration yielded membranes stripped of their ribosomes with retention of activity for translocation and processing. Stripped microsomal membranes showed a selective concentration of approximately 25 polypeptides in the membranes when analysed by two-dimensional polyacrylamide-gel electrophoresis. The two-dimensional electrophoretic profile was catalogued into polypeptides that are glycoproteins, those that contain free thiol groups disposed at the cytosolic surface of microsomal vesicles and those that are of secretory origin but have been entrapped in the microsomal preparation. Several secretory components, including amylase, procarboxypeptidases, lipase and anionic trypsinogen, were tentatively identified among the microsomal polypeptides. The rough and stripped microsomal membranes from dog pancreas show a characteristic set of seven major acidic polypeptides, which are also identifiable in microsomal-membrane preparations isolated from dog liver and rat liver. One of these polypeptides was identified as protein disulphide-isomerase (EC 5.3.4.1).

The pancreatic rough endoplasmic reticulum is a highly specialized subcellular microsomal organelle containing membrane-bound polyribosomes engaged in the synthesis and vectorial translocation of nascent secretory digestive enzymes (Palade, 1975) and in proteolytic processing and other post-translational modifications of newly synthesized polypeptides. In recent years some insight into the mechanisms of association and insertion of nascent polypeptides into endoplasmic-reticulum membranes has emerged from identification and characterization of several microsomal-membrane proteins. Blobel and co-workers have purified an 11S particle, termed a signal-recognition complex, composed of six polypeptides and a 7S RNA molecule, from rough microsomal membranes, and have shown that the

complex selectivity arrests elongation of secretory polypeptides synthesized on free polyribosomes (Walter *et al.*, 1981). Dobberstein's group have identified an elastase-sensitive peripheral microsomal-membrane protein of M_r approx. 70 000 that has strong affinity for the signal-recognition complex and when docked on to by signal-recognition-complex-bound polyribosomes relieves the elongation arrest of the nascent polypeptide imposed by that particle (Meyer *et al.*, 1982). Two integral membrane polypeptides, ribophorins, of M_r approx. 65 000 and 63 000, in rat liver microsomal fraction have been characterized by Kreibich *et al.* (1978) and have been implicated to act as ribosome receptors in rough microsomal membranes. For a greater understanding of the secretory functions and other specialized activities such as lipid biosynthesis, associated with pancreatic endoplasmic reticulum, a comprehensive analysis of the polypeptides that constitute this membrane is essential.

Abbreviations used: SDS, sodium dodecyl sulphate; 1,5-I-AEDANS, *N*-iodoacetyl-*N'*-(5-sulpho-1-naphthyl)-ethylenediamine.

The most powerful and widely applicable technique for resolving a large number of distinct polypeptides is the two-dimensional method of O'Farrell (1975). Although this procedure has proved to be of limited application in resolving polypeptides from many membrane systems, owing to their limited solubility in the non-ionic detergents and high concentrations of urea used in the first dimension, modifications in the sample preparation have made O'Farrell's method fully applicable to resolving most of the polypeptides occurring in liver microsomal membranes (Kaderbhai & Freedman, 1980). By this approach a detailed picture of the polypeptides constituting liver microsomal membranes, their quantity, their inducibility, their spatial disposition and their topographical features is already beginning to emerge (Vlasuk & Walz, 1980).

In the present paper we report on the polypeptide composition of dog pancreatic rough and stripped microsomal membranes, both of which are shown to possess ability to segregate and process proteins translated *in vitro* from bovine pituitary prolactin mRNA. We show that the two-dimensional polyacrylamide-gel electrophoretic technique can be applied to resolve about 110 distinct polypeptides from rough pancreatic microsomal membranes, leaving no aggregated material at the point of sample application in the first-dimensional separation. By use of this powerful technique, coupled with peptide mapping, we have characterized (i) seven major acidic polypeptides that appear to be common to dog pancreas, dog liver and rat liver microsomal membranes, (ii) a post-translational modifying enzyme, namely protein disulphide-isomerase, (iii) pancreatic secretory components entrapped in microsomal vesicles, (iv) glycoproteins and (v) proteins containing free thiol groups disposed at the cytoplasmic surface of the microsomal membranes.

Experimental procedures

Chemicals

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Co., Poole, Dorset, U.K., and BDH Chemicals, Poole, Dorset, U.K.

Preparation of microsomal membranes

Dog pancreatic microsomal membranes were prepared by the method of Blobel & Dobberstein (1975) with a few modifications. The animals were pre-administered with 2 dog units of pancreozymin/cholecystokinin (Boots, Nottingham, U.K.)/kg body wt., under anaesthesia, before excision of the pancreas. The organ was quickly removed from the animal, washed extensively

in chilled homogenizing buffer A [0.25M-sucrose in 25mM-KCl/5mM-MgCl₂/50mM-Tris/HCl buffer, pH 7.5, containing 2mM-dithiothreitol, 45 µg of phenylmethanesulphonyl fluoride (BDH Chemicals)/ml and 1000 units of Trasylol (Bayer, Surbiton, Surrey, U.K.)/ml]. All subsequent operations were conducted at 4°C. After the connective tissue had been dissected away, the gland was minced with a razor and passed through a tissue press. The resulting pulp was homogenized (1 g/ml) in buffer A in a Potter-Elvehjem homogenizer unit driven by a motor at 400 rev./min with ten complete passes. A post-mitochondrial supernatant was obtained by centrifugation of the homogenate at 13000 *g*_{av.} for 10 min. The supernatant was applied to a Sepharose CL-2B (Pharmacia, London W.5, U.K.) column (4.5 cm internal diam. × 20 cm) (Hawkins & Freedman, 1979) that had been pre-equilibrated with buffer A without proteinase inhibitors. The crude membrane fraction eluted in the void volume was collected, and the retarded peak, consisting of soluble material, was discarded. Rough microsomal membranes were obtained by layering the gel-filtered membranes over a discontinuous sucrose gradient composed of 6 ml of 1.65M-sucrose in TMK buffer (5mM-MgCl₂/25mM-KCl/50mM-Tris/HCl buffer, pH 7.5), which in turn was layered over 6 ml of 2.1M-sucrose in TMK buffer and centrifuged at 130000 *g*_{av.} for 3.5 h. The membranes were harvested from the 1.65M-sucrose/2.1M-sucrose interface, diluted with 3 vol. of TMK buffer and re-centrifuged at 105000 *g*_{av.} for 1.5 h. Stripped microsomal membranes were prepared by treating rough microsomal membranes with EDTA and high KCl concentration as described by Meyer & Dobberstein (1980). The final pellets obtained were resuspended in 0.25M-sucrose in 20mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer, pH 7.5, flash-frozen in liquid N₂ and stored as 100 µl portions at -70°C.

Dog liver and rat liver microsomal membranes were prepared, and from these 0.2%-(w/v)-sodium deoxycholate-solubilized extracts were obtained as described by Kaderbhai & Freedman (1980). Proteins extracted by deoxycholate were radioiodinated by the chloramine-T method (Hunter & Greenwood, 1964) to a final specific radioactivity of 8.4×10^8 c.p.m./mg of protein.

Pancreatic juice

Pancreatic juice was collected by retrograde cannulation of the pancreatic duct of a dog kept under anaesthesia with phenobarbital. During administration of 4.2 units of pancreozymin/cholecystokinin/kg body wt., the pancreatic juice was collected over a period of 2 h, frozen in solid CO₂ and subsequently stored at -70°C.

Two-dimensional polyacrylamide-gel electrophoresis

Two-dimensional polyacrylamide-gel electrophoresis was performed as described by O'Farrell (1975) with the following modifications. The first-dimension gels contained Triton X-100 and NP-40 each at 1% (w/v) concentration to give a broader solubilizing power for proteins that may be sparingly soluble in just one detergent alone. The pH gradient in the first dimension was constructed by using LKB (Bromma, Sweden) Ampholine pH 3.5–10 mixture at a final concentration of 2% (w/v) in the isoelectric-focusing gel. The isoelectric-focusing gel was cast in 140 mm-long tubes with internal diameter 4.6 mm to a height of 12 cm. To minimize cathodic drift caused by loss of Ampholines during electrofocusing, the bottoms of the tubes were sealed with a square piece of dialysis membrane held in position by a ring of silicone-rubber tubing. Unless stated otherwise in the text, the following protocol was followed for sample preparation. A 100 μ l portion of microsomal membrane suspension at 20 mg/ml protein concentration in 0.25 M-sucrose/20 mM-Hepes/KOH buffer, pH 7.5, was diluted with 90 μ l of water. To this 10 μ l of ribonuclease A (4 mg/ml) was added, and the mixture left to stand at room temperature for 30 min to digest RNA. Membranes were solubilized by addition of 400 μ l of lysis buffer composed of 10 M-urea, 2% (w/v) Triton X-100, 2% (w/v) NP-40, 7.5% (v/v) 2-mercaptoethanol, 2% (w/v) Ampholines (pH 3.5–10, pH 4–6 and pH 6–8, in the proportions 1:2:2) and left to stand for a further 30 min period.

The sample was then saturated with urea by adding 200 μ g of solid urea. After preparation, the sample was used immediately in order to avoid any charge modification of the native proteins. Unless stated otherwise, samples contained in 80–100 μ l were normally applied at the acidic ends of the prefocused isoelectric-focusing gels. The pH gradient in the first dimension was monitored as a routine by slicing the isoelectric-focused gel into 1 cm pieces, soaking each of them in 1 ml of distilled water overnight at 4°C, and determining the pH of the solution. The second-dimensional separation was carried out in a linear polyacrylamide slab-gel 16–7% gradient containing 0.1% SDS throughout and at pH 8.5 (Johnson, 1982). Myosin (M_r 220 000), β -galactosidase (M_r 130 000), phosphorylase b (M_r 94 000), serum albumin (M_r 68 000), ovalbumin (M_r 45 000) and carbonic anhydrase (M_r 30 000) were used as the marker proteins to calibrate the second-dimensional gels. After electrophoresis the gels were stained with Coomassie Blue R-250 as described previously (Kaderbhai & Freedman, 1980), or with silver stain by the method of Wray *et al.* (1981).

Identification of thiol-containing proteins

Intact microsomal vesicles at 20 mg/ml protein concentration in 0.25 M-sucrose/20 mM-Hepes/KOH buffer, pH 7.5, were incubated with 1 mM-1,5-I-AEDANS (Kaderbhai *et al.*, 1982) for 10 min at room temperature. After inactivation of excess 1,5-I-AEDANS by addition of L-cysteine to a final concentration of 10 mM, 1,5-I-AEDANS-modified microsomal membranes were sedimented and resuspended in fresh buffer. The membrane polypeptides were prepared for isoelectric focusing as described above and resolved in two-dimensional polyacrylamide-gel electrophoresis. Immediately after completion of electrophoresis, fluorescent spots were detected and photographed under illumination by u.v. light.

Glycoprotein identification

Glycoproteins were identified by their affinity for 125 I-labelled concanavalin A in the presence or in the absence of α -methyl mannoside as described by Burridge (1976).

Translation and processing in vitro

Bovine pituitary polyadenylated RNA [RNA purified by affinity chromatography on oligo(dT)-cellulose] (50 μ g/ml) was translated in a ribonuclease-treated rabbit reticulocyte lysate in the presence or in the absence of microsomal membranes as described by Shields & Blobel (1978).

Peptide fingerprint analysis

Peptide mapping was carried out by limited proteolysis with *Staphylococcus aureus* proteinase in SDS/polyacrylamide gels (15%, w/v) as described by Cleveland *et al.* (1977).

Results and discussion

Characterization of dog pancreatic microsomal-membrane preparation

Sections of the preparation of dog pancreatic rough microsomal membranes when examined under the electron microscope were found to consist of characteristic intact vesicular elements ranging in size from approx. 200 to approx. 600 μ m in diameter. The rough-microsomal preparation was entirely composed of vesicles studded with ribosomes. No plasma-membrane- or mitochondria-related structures were observed. Similar ultrastructures have been observed in preparations of rough microsomal membranes examined in other laboratories (Palade & Siekevitz, 1956). On treatment of rough microsomal membranes with EDTA and high KCl concentrations ribosomes were detached, and the re-isolated membranes examined under the electron microscope exhibited

Fig. 1. Densitometric scans of fluorograms showing translation products formed *in vitro* directed by affinity-purified bovine pituitary mRNA and its processing by isolated dog pancreatic microsomal membrane

Radiolabelled proteins were biosynthesized in the presence of [^{35}S]methionine in a ribonuclease-treated rabbit reticulocyte-lysate translation system for 90 min at 30°C. The products were separated by SDS/polyacrylamide-gel (15%, w/v) electrophoresis and detected by fluorography on a Kodak X-Omat X-ray film that was scanned on a Kratos SD 3006 spectrodensitometer. Translations were carried out in the presence of (1) bovine pituitary mRNA alone at 50 $\mu\text{g}/\text{ml}$, or together with (2) and (3) ribonuclease-treated rough microsomal membranes at 5.6 and 2.8 A_{260} units/ml respectively, and (4) microsomal membranes, degranulated with EDTA and high KCl concentration, at 5.0 A_{260} units/ml.

gross degranulated smooth vesicular appearance. Chemical determination of RNA and protein content of the isolated fractions showed that on degranulation the RNA/protein ratio by weight was decreased from 0.17 to 0.10.

The preparations of rough microsomal membranes that had been pretreated with micrococcal nuclease to destroy endogenous mRNA, and rough microsomal membranes that were stripped of ribosomes by treatment with EDTA and high KCl concentration, were both active in their ability to process preprolactin when added to rabbit reticulocyte translation systems programmed with bovine pituitary polyadenylated RNA (Fig. 1), in the assay system *in vitro* developed by Shields & Blobel (1978). The presence of microsomal membranes during the translational phase resulted in the conversion of preprolactin, of M_r 26000, into mature prolactin, of slightly lower M_r .

Two-dimensional polyacrylamide-gel electrophoresis of dog pancreatic microsomal membrane polypeptides

Fig. 2 shows a typical resolution in two-dimensional polyacrylamide-gel electrophoresis of dog pancreatic rough-microsomal-membrane polypeptides loaded from the acidic end of the isoelectric-focusing gel in the first dimension. The electrophoretogram displays a complex profile of about 110 distinct polypeptides detected by Coomassie Blue staining. The number of polypeptides increased to over 200 when the more sensitive silver staining procedure was used for detecting the polypeptides. The two-dimensional electrophoretic pattern shows nine polypeptides that stain relatively intensely with Coomassie Blue, of which the six components (numbered 1, 3, 5, 7, 8 and 12), although differing in their M_r values, significantly migrated mainly to the acidic region of the isoelectric-focusing gel and all have pI 5 or very close to this value. The most acidic polypeptide in the profile, numbered 11, with M_r 52000 and pI approx. 4.2, stains weakly with Coomassie Blue, but the protein is radioiodinated readily and appears as an intensely labelled spot when detected by autoradiographic procedures. A comparison of the two-dimensional electrophoretic profile of microsomal-membrane polypeptides with a separation of a similar sample solubilized with SDS and reduced with 2-mercaptoethanol, run only in the second dimension, SDS/polyacrylamide-gel electrophoresis (see Fig. 2), shows that, of the polypeptides that stain with Coomassie Blue, few, if any, are lost from the samples applied to the first-dimensional isoelectric-focusing gel.

For good and reproducible resolution of the microsomal-membrane polypeptides it was found essential to treat the rough microsomal membranes with ribonuclease before the addition of lysis

Fig. 2. Dog pancreatic rough-microsomal-membrane polypeptides resolved by two-dimensional polyacrylamide-gel electrophoresis

Approx. 200 μ g of sample protein was loaded at the end of the first-dimensional gel and subjected to isoelectric focusing followed by second-dimensional separation by SDS/polyacrylamide-gel electrophoresis in a slab gel in a vertical direction. The polypeptides were detected by staining with Coomassie Blue. The left-hand track shows an identical sample resolved in one-dimensional SDS/polyacrylamide-gel electrophoresis alone (120 μ g). The right-hand track shows M_r markers.

buffer. Omission of ribonuclease treatment consistently gave rise to aggregation at the sites of loading from either the acidic or the basic end of the isoelectric-focusing gels, allowing only small proportions of the protein loaded to penetrate the gel matrix and be resolved successfully. After ribonuclease treatment of the microsomal membranes, two-dimensional polyacrylamide-gel electrophoresis gave reproducible spot patterns irrespective of the site of loading in the isoelectric-focusing gel, with the exception of two additional acidic polypeptides numbered 10 and 42, which appeared in the protein maps after loading from the basic end (see Fig. 5c). The high degree of reproducibility in the resolution suggests the absence of heterogeneity arising as an artifact from charge modification. It is unlikely that the polypeptide profile exhibits any major polypeptides arising from cellular organelles other than microsomal membranes, which appeared to be homogeneous under the electron microscope. It is likely that soluble proteins would have been desorbed from the microsomal preparation during passage through Sepharose CL-2B (Tangen *et al.*, 1973).

In order to eliminate proteins of ribosomal origin from the two-dimensional electrophoretic profile, rough microsomal membranes were stripped by treatment with EDTA and high KCl concentration and the smooth microsomal membranes re-isolated. The two-dimensional electrophoretic polypeptide profile of stripped microsomal membranes (Fig. 3) was much less complex, comprising about 52 polypeptides detected by silver staining. The ribosomal proteins, which are highly basic and of M_r below 45000 (Sherton & Wool, 1972), appear as streaky material in Fig. 2, but are deleted from the profile by the stripping procedure. Several large polypeptides, including those numbered 2, 4, 13 and 23 (Fig. 2), and many other minor polypeptides are also removed by stripping, and it may be deduced that these polypeptides are not essential for the activity *in vitro* that the microsomal membranes exhibit in segregating and processing secretory proteins. A major acidic polypeptide (no. 5), which has been shown to bear carbohydrate (see below), gives rise to a doublet spot, but the nature of the charge modification remains to be elucidated. A number

Fig. 3. Two-dimensional polyacrylamide-gel electrophoresis of dog pancreatic stripped-microsomal-membrane polypeptides. Rough microsomal membranes were degranulated and the sample was prepared for isoelectric focusing as described in the Experimental procedures section. Approx. 15 μ g of protein was loaded from the acidic end. The polypeptides are silver stained.

of polypeptides, including those numbered 19, 21, 22 and 26, and a group of five pairs of polypeptides (numbered 29–38), each pair possessing slightly differing pI values and each member of the pair differing from the other member by about M_r 1000, appear as more prominent spots in the two-dimensional profile of stripped microsomal membranes than in the two-dimensional profile of rough microsomal membranes, suggesting that these components are selectively concentrated in the microsomal membranes by degranulation. Polypeptides 29–38 are also selectively extracted by deoxycholate (see below).

Previous analyses by SDS/polyacrylamide-gel electrophoresis in one dimension of rat liver microsomal membranes resolved about 54 polypeptides (Sharma *et al.*, 1978). Stripping of liver rough microsomal membranes of their ribosomes was reported to lead to an increase in prominence of polypeptides of M_r approx. 27 000, 32 000, 33 000, 40 000, 63 000 and 87 000 in the residual membranes, as well as the expected removal of ribosomal polypeptides. Polypeptides in pancreatic microsomal membranes have been found previously to possess common immunological characteristics with rat liver ribophorins, but differ somewhat in electrophoretic mobility on SDS/polyacrylamide-gel electrophoresis (Marcantonio *et al.*, 1982). We have not yet been able to identify ribophorins in the profiles shown in Figs. 2 and 3, where there are several potential candi-

dates for pancreatic ribophorins in the M_r range 60 000–65 000.

Pancreatic secretory proteins isolated in the microsomal-membrane preparation

In order to identify any secretory digestive enzymes that may have become entrapped in the microsomal-membrane preparation during homogenization of pancreatic tissue (Palade & Siekevitz, 1956), we have compared the two-dimensional electrophoretic profiles displayed by proteins from (i) dog pancreatic secretory juice (Fig. 4b) and (ii) dog pancreatic secretory juice to which rough-microsomal-membrane proteins had been added (Fig. 4a). Comparison of these two-dimensional profiles with the two-dimensional electrophoretic profile of rough-microsomal-membrane polypeptides alone (see Fig. 2) shows that a major secretory polypeptide occurring in pancreatic juice comigrates with a prominent polypeptide (spot 18) from microsomal membranes. We tentatively conclude that this spot is amylase, because it is the most abundant secretory protein in pancreatic juice and because its M_r and pI are quite close to those reported for purified amylase (Scheele *et al.*, 1980), and that spots 17, 20, 23 and 41 are small amounts of lipase, procarboxypeptidase A_3 , procarboxypeptidase A_1 and anionic trypsinogen trapped in the rough-microsomal-membrane preparation. Our observation that abundant digestive enzymes synthesized in the endoplasmic reticulum

Fig. 4. Two-dimensional polyacrylamide-gel electrophoresis of (a) dog rough-microsomal-membrane polypeptides (120 µg) to which pancreatic-juice polypeptides (150 µg) had been added and (b) pancreatic-juice proteins (350 µg)

GP1 and GP2 are glycoproteins 1 and 2 respectively. Although microsomal-membrane polypeptide 5 co-migrates with pancreatic-juice protein (5), structurally these polypeptides are dissimilar (see the text); polypeptides 17, 18, 20, 23 and 41 have been tentatively identified as lipase, amylase, pro-carboxypeptidases A₃ and A₁ and anionic trypsinogen (for a further comparison see Fig. 2).

Fig. 5. Deoxycholate-solubilized microsomal-membrane polypeptides from (a) rat liver, (b) dog liver and (c) dog pancreas analysed by two-dimensional polyacrylamide-gel electrophoresis

The samples were prepared as described in the Experimental procedures section. Approx. 200 µg of protein was loaded from the basic end of the gel. pA and A are proalbumin and albumin respectively; spot 18 is amylase. The corresponding spots appearing to be identical in the three sources of microsomal membranes are numbered.

are trapped in pancreatic microsomal preparation is similar to previous and present observations that albumin and proalbumin become trapped in liver microsomal preparations (Vlasuk & Walz, 1982; Figs. 5a and 5b).

Glycoproteins occurring in dog pancreatic rough microsomal membranes were identified after resolution in two-dimensional polyacrylamide-gel electrophoresis by their affinity for ¹²⁵I-labelled concanavalin A (Table 1). No binding of ¹²⁵I-labelled concanavalin A to these glycoproteins was observed in the presence of α-methyl manno-

side, implying that the observed binding is specific for carbohydrate moieties. Glycoproteins were also identified among the pancreatic-juice polypeptides resolved in the two-dimensional electrophoresis. By this approach microsomal-membrane polypeptide 17, which co-migrates with secretory lipase (Fig. 4), is shown to be a glycoprotein (Table 1), as reported previously for human pancreatic lipase (Caro *et al.*, 1977). In addition, a major glycoprotein present in rough and smooth microsomal membranes (no. 5, M_r 100 000, pI 4.9) co-migrates with a similar secretory glycoprotein, but the

Fig. 6. Peptide fingerprint analysis of *S. aureus* V₈ proteinase digests of corresponding polypeptide spots eluted from two-dimensional electrophoresis gels of rat liver (RL), dog liver (DL) and dog pancreatic (DP) microsomal membranes and dog pancreatic juice (PJ). Each of the protein spots eluted was partially digested with 20 ng of the proteinase, and peptide fragments were resolved on a 15% homogeneous gel and detected by silver staining.

eluted spots yield different peptide fingerprints (see tracks 6 and 7 in Fig. 6), suggesting that their co-migration on two-dimensional electrophoresis was fortuitous.

Although a number of the major polypeptides of dog pancreatic microsomal membranes analysed in the present study are shown to be glycoproteins, the structure and function of protein-bound carbohydrate moieties in microsomal-membrane polypeptides has not been fully investigated. Boulan *et al.* (1978) have suggested that lectin-binding sites are exposed mainly on the luminal surface of both rough and stripped microsomal membranes from rat liver. In contrast, other workers have presented evidence that the carbohydrate moieties are both lumenally and cytoplasmically disposed (Bergman & Dallner, 1976). There are indications that some microsomal enzymes and ribophorins are glycoproteins (Evans & Gurd, 1973; Bischoff *et al.*, 1975; Grebenau *et al.*, 1977).

Identical polypeptides in pancreatic and liver microsomal-membrane preparations

A comparison of the two-dimensional polyacrylamide-gel-electrophoretic profile of dog pancreatic rough-microsomal-membrane polypeptides (Fig. 2) with those of liver microsomal-membrane polypeptides, previously published, shows that the pancreas clearly lacks the basic polypeptides constituting the drug-metabolizing enzymes (cytochrome *P*-450 species, epoxide hydratase etc.) observed in liver microsomal preparations (Vlasuk *et al.*, 1982). The acidic polypeptides numbered 1, 3, 5, 7, 8, 12 and 13 present a consistent and easily identifiable constellation of spots. These polypeptides are present in the membranes at high concentrations and have pI values that are close to each other (pI approx. 5.50). In previous work (Kaderbhai & Freedman, 1980) a similar constellation of acidic polypeptides was also resolved by two-dimensional polyacrylamide-gel electrophoresis of rat liver microsomal-membrane polypeptides.

To identify the microsomal-membrane polypeptides that are common to liver and pancreas, two-dimensional polyacrylamide-gel electrophoresis of deoxycholate extracts of dog pancreatic, dog liver and rat liver microsomal-membrane polypeptides were compared (Fig. 5). In addition, co-electrophoresis in two dimensions of ^{125}I -labelled proteins ($5\mu\text{g}$) from deoxycholate extracts of either dog pancreatic or rat liver microsomal preparations was performed. Dog pancreatic or rat liver microsomal-membrane proteins were detected by Coomassie Blue staining, whereas the dog liver microsomal-membrane polypeptides were detected by autoradiography of the same dried gel. Comparisons were made with gels obtained by loading proteins from either the

acidic or the basic end of the isoelectric-focusing gels. Superimposition of the autoradiograph on to the Coomassie Blue-stained profile permitted detection of those polypeptides that are common (Table 1). The most prominent polypeptides that co-migrated in the two-dimensional electrophoresis of dog pancreatic, dog liver and rat liver microsomal-membrane polypeptides were the acidic components 3, 5, 7, 12, 13 and 15. Polypeptide 11 is seen readily on autoradiographs, but stains weakly with Coomassie Blue, and is found in dog liver and dog pancreatic microsomal membranes but not in rat liver microsomal membranes. Polypeptides 10 and 42 are common to microsomal membranes from the three sources (Fig. 5), but are resolved only after loading from the basic end of the isoelectric-focusing gel, and thus appear faintly stained in Fig. 2 as the separation shown in this gel was performed by loading from the acidic end. Minor polypeptide components common to dog pancreatic and dog liver microsomal membranes are also listed in Table 1. The major acidic polypeptide 8, present in dog pancreatic microsomal membranes, was clearly absent from dog liver and rat liver microsomal membranes. For a further confirmation that the co-migrating acidic polypeptides from rat liver, dog liver and dog pancreatic microsomal membranes were structurally identical, the peptide maps of the corresponding polypeptides from the three sources were examined after limited proteolysis with *S. aureus* V₈ proteinase (Fig. 6). Although there are some variations in the peptide maps of corresponding microsomal polypeptides 3, 5, 7, 10, 12 and 13 in the positions of minor bands, after taking into consideration differences in loading and intensity of staining the overall correspondence of the majority of the fragments indicates that the analogous numbered polypeptides from different sources are structurally related. Variations in the peptide fingerprints are most conspicuous when comparisons are made between the fingerprint maps derived from polypeptides from different species rather than different organs of the same species. Polypeptide 15 from dog pancreas and that from dog liver yield comparable fingerprints, thus suggesting some close structural similarity in these polypeptides from the two organs. The corresponding polypeptide 15 from rat liver shows five peptide fragments that co-migrate, but the structural relatedness is not as clearly obvious to that seen in the polypeptide 15 from dog pancreas and dog liver.

Identification of protein disulphide-isomerase as a component of dog pancreatic microsomal membrane

The close resemblance of the pI and M_r values of polypeptide 12 in dog pancreatic microsomal-membrane polypeptides resolved by two-dimen-

Table 1. *Characterization of polypeptides in dog pancreatic rough and stripped microsomal membranes*
For full experimental details see the text.

Polypeptide no.	Relative quantification by staining in rough membranes*	Presence of protein in stripped membranes†	Presence of glycoprotein‡	Presence of SH-containing protein§	Presence of secretory component	M _r	pI (approx.)	Identity	Presence of identical protein in liver microsomal membranes
1	++	+	+	-	-	190000	4.9	-	-
2	++	-	-	-	-	175000	6.5	-	-
3	+++	+	+	-	-	160000	5.1	-	+
4	++	-	-	-	-	117000	6.9	-	-
5	+++	+	+	-	+	100000	4.9	Glycoprotein 1?	+
6	+	+	-	-	-	100000	5.2	-	-
7	+++	+	-	-	-	78000	5.0	-	+
8	+++	+	+	+	-	70000	4.8	-	-
9	++	-	+	-	+	72000	5.7	-	-
10	+++	+	-	-	-	60000	4.3	-	+
11	+	-	-	-	-	52500	4.2	-	+
12	+++	+	-	+	-	57500	5.0	Protein disulphide-isomerase	+
13	++	-	-	+	-	54000	5.05	-	+
14	+	-	-	+	-	58000	5.4	-	-
15	+++	+	-	+	-	60000	5.9	-	+
16	++	-	-	-	-	59000	6.1	-	-
17	++	-	+	-	+	57000	6.2	Lipase	-
18	+++	+	-	-	+	58000	6.7	Amylase	-
19	+	+	+	-	-	79000	6.5	-	-
20	+	-	-	-	+	51000	5.7	Procarboxypeptidase A ₃	-
21	+	+	-	-	-	49000	7.8	-	-
22	+	+	-	-	-	49000	7.6	-	-
23	+	-	-	-	+	47500	6.6	Procarboxypeptidase A ₁	-
24	+	+	-	+	-	49500	5.2	-	+
25	+	+	-	-	-	47000	4.75	-	-
26	+	-	-	-	-	33000	7.6	-	-
27	+	-	+	-	-	29500	7.4	-	-
28	+	-	-	-	-	28000	7.3	-	+
29	+	+	-	-	-	32000	6.6	-	+
30	+	+	-	-	-	30000	6.75	-	-
31	+	+	-	-	-	32000	6.3	-	-
32	+	+	-	-	-	31500	6.4	-	-
33	+	+	-	-	-	32000	6.2	-	+
34	+	+	-	-	-	31000	6.2	-	-
35	-	+	-	-	-	32000	6.1	-	-
36	-	+	-	-	-	31000	6.1	-	-
37	+	+	-	-	-	34000	5.65	-	-

Table 1 (continued)

Polypeptide no.	Relative quantification by staining in rough membranes*	Presence of protein in stripped membranes†	Presence of glycoprotein‡	Presence of free SH-containing proteins§	Presence of secretory component	M_r	pI (approx.)	Identity	Presence of identical protein in liver microsomal membranes
38	+	+	—	—	—	32000	5.6	—	—
39	+	+	—	—	—	26000	6.6	—	—
40	+	+	—	—	—	25000	5.7	—	—
41	+	—	—	—	+	30000	4.6	Anionic trypsinogen	—
42	+	—	—	—	—	88000	4.7	—	—

* Based on affinity for Coomassie Blue R-250.

† Polypeptides detectable in membranes sedimented after treating rough microsomal membranes with EDTA and high concentration of KCl.

‡ Identified by their affinity for 125 I-labelled concanavalin A.

§ Polypeptides in intact microsomal membranes accessible to 1,5-I-AEDANS.

|| Identical polypeptide detected in dog pancreatic secretory juice.

sional polyacrylamide-gel electrophoresis with those reported previously for purified bovine liver and rat liver microsomal protein disulphide-isomerase (Freedman *et al.*, 1983) led us to characterize this polypeptide further. Fig. 7 shows the result of subjecting dog pancreatic microsomal-membrane polypeptides to two-dimensional polyacrylamide-gel electrophoresis in the presence and in the absence of purified rat liver microsomal protein disulphide-isomerase and shows that pure rat liver enzyme co-migrates with the single major acidic dog pancreatic microsomal-membrane polypeptide 12. The identity of polypeptide 12 as protein disulphide-isomerase is confirmed by a comparison of the peptide fingerprints of the corresponding spots excised from two-dimensional electrophoresis gels of rat liver, dog liver and dog pancreatic microsomal-membrane polypeptides (Fig. 5). The corresponding polypeptides isolated from these three sources gave almost identical fingerprints (Fig. 6). The abundant prominence of the polypeptide 12 in the two-dimensional electrophoretogram shows that this enzyme is abundant in dog pancreatic microsomal membranes, and the similarity of peptide fingerprints (Fig. 6) and pI values (Fig. 5) of protein disulphide-isomerase from rat liver, dog liver and dog pancreatic microsomal membranes suggests that the enzyme is structurally well conserved. Enzymic characterization of protein disulphide-isomerase in pancreas has previously proved difficult owing to the presence of high ribonuclease activity in this tissue, which interferes with the assay of protein disulphide-isomerase activity (Hillson & Anderson, 1982). The identification of protein disulphide-isomerase in dog pancreatic microsomal membranes, its structural conservation (rat, dog and bovine tissues) and its abundance in microsomal preparations suggests that the enzyme may play a role in catalysing interchange of disulphide bonds in pancreatic digestive enzymes.

Covalent labelling of microsomal polypeptides with a thiol-specific fluorescent probe

Incubation of intact rough and stripped dog pancreatic microsomal membranes with the thiol-specific fluorescent probe 1,5-I-AEDANS leads to specific labelling of some of the major acidic polypeptides (8, 12, 13, 14, 15 and 24; see Table 1), as seen under u.v. illumination. Owing to the presence of a sulphonic acid group, the reagent is likely to be membrane-impermeant (Mueller & Morrison, 1974).

Conclusion

The structural conservation of a number of acidic polypeptides in the microsomal membranes

Fig. 7. Identification of protein disulphide-isomerase (arrowed) as a component of pancreatic microsomal membranes. Dog pancreatic microsomal-membrane polypeptides (22 µg) (a) in the absence or (b) in the presence of 4 µg of purified (256-fold) rat liver microsomal protein disulphide-isomerase were subjected to two-dimensional polyacrylamide-gel electrophoresis. Spots marked S represent impurities in the preparation. Proteins were detected by silver staining.

of rat liver, dog liver and dog pancreas suggests that these polypeptides play an important role in the endoplasmic reticulum. Vlasuk & Walz (1982) have carried out extensive analysis of rat liver microsomal polypeptides, and shown that none of the acidic polypeptides appear to be induced by pretreatment of the animals with various xenobiotics. The large amounts of the acidic polypeptides present in the microsomal membranes, the closeness of their pI values and their structural and charge conservation raise a number of questions about their roles, and it is conceivable that they may be involved in lipid biosynthesis, or in segregation and co- and post-translational modification(s) of secretory proteins. Of the polypeptides common to liver and pancreatic microsomal membranes, listed in Table 1, those numbered 3, 5, 7, 10, 11, 12, 24, 29 and 37 survive stripping by treatment with EDTA and high salt concentration, and therefore may be implicated in the activity of microsomes in co-translational processing of secreted proteins *in vitro*. Polypeptide 12 is homologous to protein disulphide-isomerase purified from rat liver microsomal membranes, raising the possibility that the enzyme may be involved in catalysis of interchange of disulphide bonds of pancreatic digestive enzymes and other secretory components that are known to be rich in disulphide linkages (Jocelyn, 1972).

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