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A membrane-bound form of protein disulfide isomerase (PDI) and the hepatic uptake of organic anions

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Protein disulfide isomerase (PDI) was considered to be involved in the hepatic uptake of certain organic anions because the protein is photoaffinity labeled by photolabile derivatives of the bile acid taurocholate. Several lines of evidences including photoaffinity labeling experiments indicated a close relationship between the uptake of bile acids and the organic anion bumetanide. The possible involvement of PDI in hepatic transport processes of these organic anions was tested with polyclonal antibodies raised against a PDI-β-galactosidase fusion protein. Western blot analysis and immunofluorescence of intact hepatocytes showed that protein disulfide isomerase is located in sinusoidal rat liver plasma membranes. This protein is immunologically identical with microsomal PDI prepared from bovine liver. The plasma membrane form of PDI is, however, not labeled by photoactivated bumetanide as revealed by two-dimensional gel electrophoresis. These results indicate that, although a membrane-bound form of the PDI is present in the sinusoidal plasma membrane of rat hepatocytes, this protein is not involved in the hepatocellular uptake of the organic anion bumetanide.

Introduction

Protein disulfide isomerase (EC 5.3.4.1) is a multifunctional, intracellular enzyme which catalyses thiol–disulfide interchange reactions in protein substrates leading to net protein disulfide formation, isomerization, or reduction, depending on the initial substrates and imposed thiol–disulfide potential [1]. Protein disulfide isomerase (PDI) has a broad substrate specificity and has been used to catalyze the in vitro folding of a variety of proteins [2]. This enzyme, which is mainly located in the endoplasmic reticulum, plays not only multiple roles in the modification of nascent secretory proteins, but also has homologies to certain other well known proteins. The homology of PDI to the β-subunit of prolyl-4-hydroxylase, to the glycosylation site binding protein, and to the thyroid hormone binding protein has been well established (for a review, see Freedman [1]). PDI also is a subunit of the microsomal triglyceride transfer complex [3,4]. In this case it was shown that PDI is a catalytically active component of the transfer protein, since the dissociation of PDI from the complex coincided with a loss of the triglyceride transfer activity [4]. This indicated that PDI is required for certain transport function. In addition, Robillard and Konings [5], proposed that dithiol–disulfide interchange may play a general role in membrane-related processes such as transport, energy transduction and hormone receptor interactions. PDI also was suggested to be involved in the hepatocellular transport of certain organic anions. This hypothesis was supported by the fact that photoaffinity labeling of sinusoidal plasma membranes with radioactive labeled derivatives of the bile acid taurocholate (7,7′-azotaurocholate and azidobenzamidotaurocholate) resulted in a labeling of a peripheral 54 kDa protein [6], which was identical with PDI at the N-terminus [7].

Furthermore, it has been shown that the transport of organic anions such as bile acids and bumetanide depends upon membrane SH-groups [8,9]. To test the possible involvement of PDI in hepatocellular transport processes, we used the cDNA clone of PDI for the construction of a bacterial expression vector which produces a β-galactosidase-PDI fusion protein after

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heat induction. This fusion protein was then used for the production of polyclonal antibodies. With these antibodies it was first demonstrated by immunofloures-cence on intact hepatocytes that PDI is located in the sinusoidal plasma membrane which is a prerequisite of the proposed function. In a second step the location of binding proteins of the organic anion bumetanide and PDI was compared after two-dimensional gel electrophoresis. The data presented show that the photoaffinity labeled bumetanide binding proteins have a different location in two-dimensional gels than PDI. In addition, PDI is also present in cell lines such as AS-30D- and FAO-hepatoma cells which are deficient in bile acid and bumetanide transport. These results make it unlikely that the PDI is involved in the hepatic uptake of bumetanide and related organic anions, although the enzyme is located in the plasma membrane of hepatocytes.

Materials and Methods

Materials

[^{3}H]Bumetanide (specific activity 15 Ci/mmol; 55.5 GBq/mmol) was prepared as described previously [10]. Non-radioactive bumetanide was a generous gift of Dr. P.W. Feit, Leo Pharmaceuticals, Denmark. ^{125}I-A14-hog insulin, with a specific activity of 2064 Ci/mmol (76.37 TBq/mmol), was kindly supplied by Prof. Dr. Laube, Medizinische Poliklinik, Giessen, Germany. Leupeptine, reduced glutathione and phenylmethylsulfonyl fluoride (PMSF) as well as non-labeled marker proteins for SDS gel electrophoresis were purchased from Sigma, Deisenhofen, Germany. Ampholines (pH 3–10) were from Pharmacia, Freiburg, Germany. Ultra-pure urea for two-dimensional gel electrophoresis was purchased from GIBCO-BRL, Eggenstein, Germany. Iodoacetamide and the reagents for gel electrophoresis (acrylamide, bis-acrylamide and tetramethylethylenediamine (TEMED)) were from Serva, Heidelberg, Germany. The second antibody used for the immunofluorescence (fluorescein-isothiocyanate-conjugated) was from Sigma, Deisenhofen, Germany. The reagents for cell culture (e.g., foetal calf serum, DMEM, trypsin) were purchased from GIBCO-BRL, Deisenhofen, Germany. Restriction enzymes and DNA-modifying enzymes (ligase, Klenow-fragment of DNA-polymerase I) were purchased from GIBCO-BRL, Deisenhofen or from Boehringer, Mannheim, Germany. All other chemicals of the highest available quality were from Serva, Heidelberg, Boehringer, Mannheim and Pharmacia, Freiburg, Germany.

The PDI-cDNA clone was kindly provided by Dr. W.J. Rutter, San Francisco, USA. An aliquot of the purified T_3 binding protein from beef liver and the corresponding antiserum was a generous gift of Dr. R. Horiuchi, Maebashi, Japan.

Methods

Preparation of a recombinant β-galactosidase-PDI fusion protein for immunization

An internal fragment of the PDI-cDNA clone [11] was subcloned into the bacterial expression vector pEX1 [12]. The cDNA clone for PDI was chosen in order to get an antibody which is highly specific for the PDI from rat. For this purpose the PDI-cDNA clone was digested with the restriction enzyme *Eco*RI (at position 1219 of the clone) and the 5′ overhang was filled in with the large fragment of DNA-polymerase I. After digestion with *Dpn*I, the resulting 324 bp fragment (between position 1219 and 1544 of the PDI clone) was isolated by preparative agarose gel electrophoresis and cloned into the pEX1 vector which was linearized with *Sma*I. The correct orientation of the resulting subclone pEX-PDI 324 was estimated by restriction-enzyme analysis. The subclone pEX-PDI 324 was then used for the preparative isolation of the fusion protein (500 ml culture volume). For further purification of the protein, the recombinant protein was isolated by preparative SDS gel electrophoresis and then used for the immunization of rabbits. After 8 to 12 weeks the rabbits were killed and the serum was prepared. The titer of the serum was 1:5000 as estimated by dot blots and Western blotting.

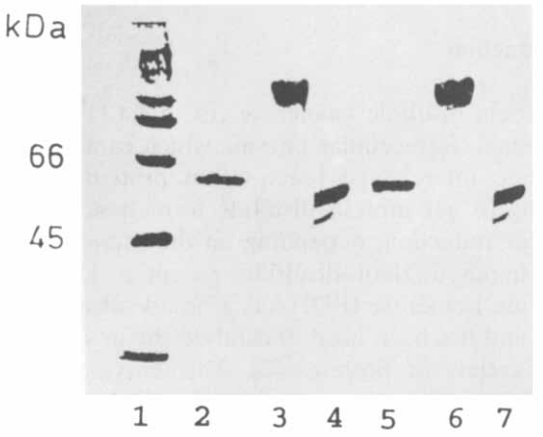

Fig. 1. Determination of the specifity of the β-galactosidase-PDI fusion protein and the resulting antiserum by Western blot analysis. Aliqouts (100 ng) of the different proteins were separated by SDS gel electrophoresis and then transferred to nitrocellulose. The lanes 2–4 were then incubated with anti-beef T_3 binding protein serum at a dilution of 1:1000. The lanes 5–7 were incubated with anti-PDI antibody at a dilution of 1:4000. Lane 1: 5 μg molecular mass marker proteins with molecular masses of 29 kDa, 45 kDa, 66 kDa, 97 kDa and 116 kDa. Lane 2: 100 ng T_3 binding protein. Lane 3: 100 ng β-galactosidase-PDI fusion protein. Lane 4: 100 ng bovine microsomal PDI. Lane 5: 100 ng T_3 binding protein. Lane 6: 100 ng β-galactosidase-PDI fusion protein. Lane 7: 100 ng bovine microsomal PDI.

Isolation of PDI from bovine liver

PDI was prepared from bovine liver according to Hillson et al. [13]. In brief, bovine liver was homogenized in 100 mM sodium phosphate (pH 7.5), 5 mM EDTA and 1% (v/v) Triton X-100. The homogenate was centrifuged at 18000 × *g* for 30 min at 4°C. The supernatant was heated in a 70°C water bath to 54°C and kept at this temperature for 15 min under continous stirring. The extract was then transferred to an ice water bath and subsequently centrifuged at 18000 × *g* for 40 min at 4°C. After ammonium sulfate fractionation (55%–85% ammonium sulfate) the final pellet was resuspended in 25 mM citrate buffer (pH 5.3). The dialysed extract was then applied to a CM-Sephadex C-50 (18 × 5 cm) column. The PDI activity containing fractions were pooled and after precipitation with ammonium sulfate (100% saturation) the resuspended and dialysed protein (in 20 mM phosphate buffer (pH 6.3)) was applied to a DEAE-Sephacel column (20 × 2.5 cm) and eluted in 20 mM phosphate buffer (pH 6.3) with a linear gradient of 0 to 0.7 M NaCl. The PDI containing fractions were pooled and dialysed against 50 mM NH_4HCO_3, and were then freeze dried.

Determination of PDI activity

PDI activity was assayed for by the ability to degrade insulin by a modification of the method originally described by Tomizawa and Halsey [14]. Up to 25 μl of PDI-containing solution was diluted with 2 mM reduced glutathione, 5 mM EDTA and 0.2% BSA to a total volume of 250 μl in 100 mM sodium phosphate buffer (pH 7.5). A mixture of 250 μl [^{125}I]insulin (4.42 nCi) and carrier insulin (0.1 mg/ml) was added and the reaction mixture was incubated for 20 min at 25°C. The reaction was stopped by addition of ice-cold 5% TCA. After 30 min at 0°C the samples were centrifuged at 10000 × *g* for 10 min TCA-soluble radioactivity was determined in the supernatant.

TABLE I

Enrichment of the specific PDI activity during the different purification steps

See also the Materials and Methods.

Purification step	Total activity (U *)	Total protein (mg)	Specific activity (mU/mg protein)
Homogenate	182	76986	2.4
Heat treatment	460	17805	26
55–85% $(NH_4)_2SO_4$	290	3864	75
CM50 eluate	167	323	519
DEAE eluate	47	33	1424
Lyophilisate	23	33	709

* Radioactivity in the supernatant fraction was corrected for approximately 2% of total radioactivity that was recovered in the supernatant fraction in the absence of the enzyme. Enzymatic activity is expressed as the corrected percentage of radioactivity in the supernatant fraction. Thus, one mU is defined as the activity that renders 20% of the total radioactivity of [^{125}I]insulin soluble in trichloracetic acid under the condition described.

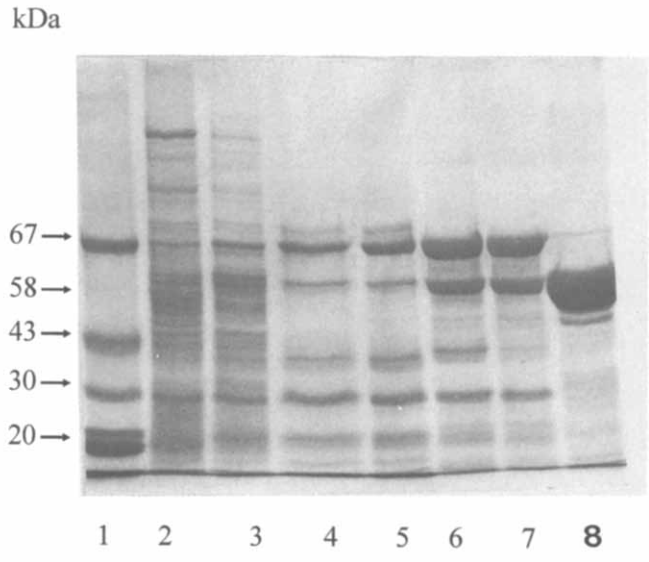

Fig. 2. SDS gel electrophoresis of aliquots of the protein from the different purification steps for PDI from bovine liver. Aliquots of the protein (10 μg per lane) from the different fractions were run on an 8% SDS gel. Shown is a Coomassie blue staining of the gel. Details of the purification steps are given in the methods section. Lane 1: marker proteins with molecular masses of 67 kDa, 43 kDa, 30 kDa and 20 kDa. Lane 2: bovine liver homogenate. Lane 3: supernatant after heat treatment of the extract. Lane 4: 55% ammonium sulfate supernatant. Lane 5: 85% ammonium sulfate pellet. Lane 6: CM50 eluate. Lane 7: 100% ammonium sulfate pellet. Lane 8: DEAE eluate.

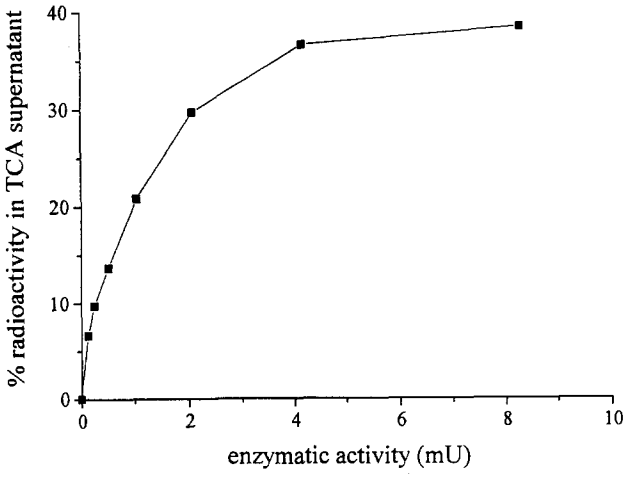

Fig. 3. Determination of the specific activity of the isolated PDI from bovine liver. Aliquots of the isolated PDI were assayed for the ability to degrade insulin according to Tomizawa et al. [14]. The PDI with glutathione was incubated for 20 min at 25°C in the presence of iodinated insulin and after the reaction was stopped with ice-cold 5% TCA, the TCA-soluble radioactivity was determined in a Pharmacia liquid scintillation counter.

Preparation of hepatocytes

Hepatocytes were isolated from male Wistar rats (230–260 g) as described previously [10]. This method based on the collagenase perfusion technique originally described by Berry and Friend [15]. In brief, the liver of anesthetized rats were perfused with Ca^{2+}-free Krebs-Henseleit buffer and 0.05% collagenase. The cells were dissociated from the tissue by bubbling carbogen into the flask yielding a crude cell suspension. By centrifugations at $50 \times g$ for 2–5 min cell debris and non-parenchymal cell were removed and the hepatocytes were resuspended in Tyrode buffer. 80–90 percent of the cells were vital as estimated by Trypan blue.

Preparation and photoaffinity labeling of rat liver sinusoidal plasma membranes

Preparation of sinusoidal plasma membranes and the photoaffinity labeling of these proteins was done as described by Petzinger et al. [16]. In brief, plasma membranes were prepared according to Blitzer and Donovan [17] on a sucrose-percoll gradient with a 20–25-fold enrichment of Na^+/K^+-ATPase activity,

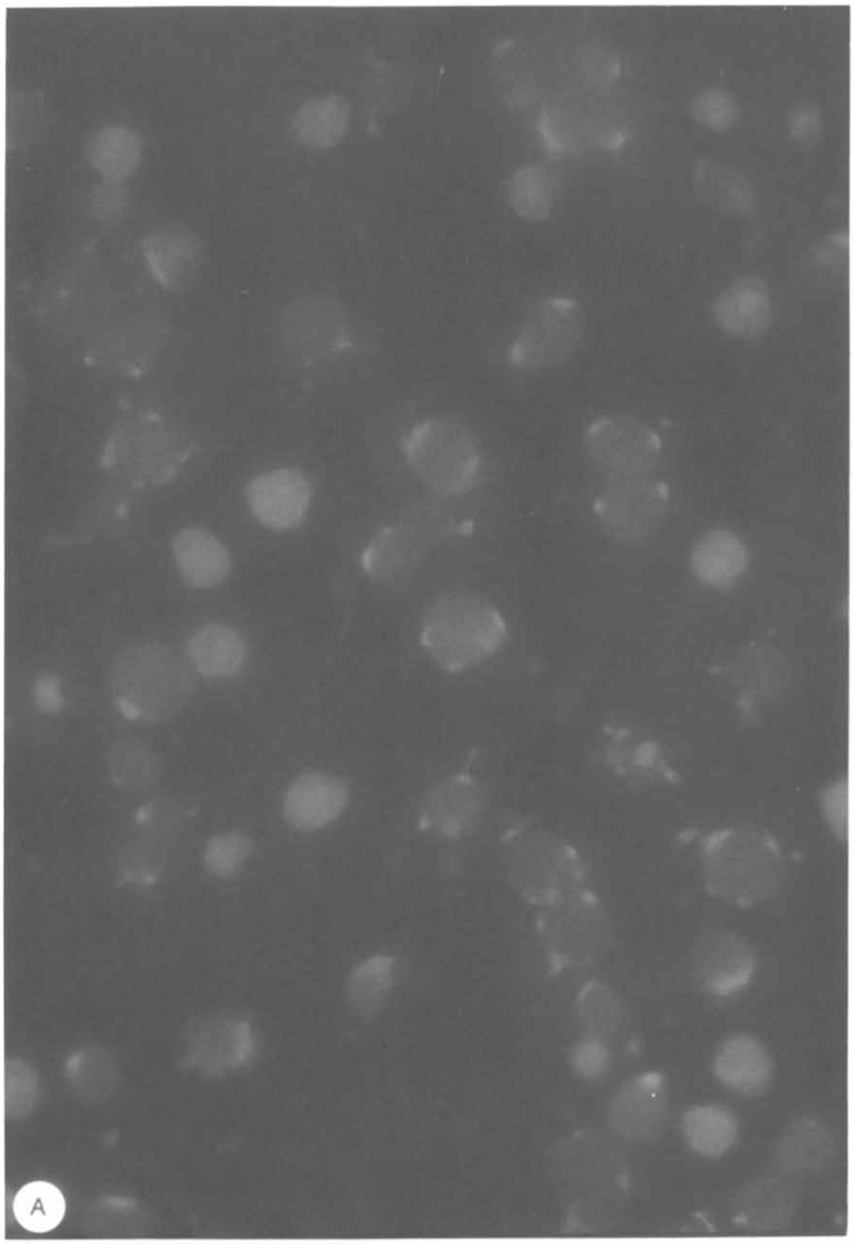

Fig. 4. Immunofluorescence on intact hepatocytes. Freshly isolated hepatocytes were grown on 12 mm glass cover slips and fixed in paraformaldehyde. After washing with PBS (pH 7.4) the cells were incubated with anti-PDI antibody at a dilution of 1:200 (18 h at 4°C). For the control, Fig. 4B, instead of the antibody only the pre-immune serum was used. After washing, the cells were incubated with fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG's at a dilution of 1:200 for 2 h. After another washing, the cover slips were mounted on slides with a glycerol-based medium and the hepatocytes were visualized with a Zeiss Universal microscope, equipped with fluorescence filter.

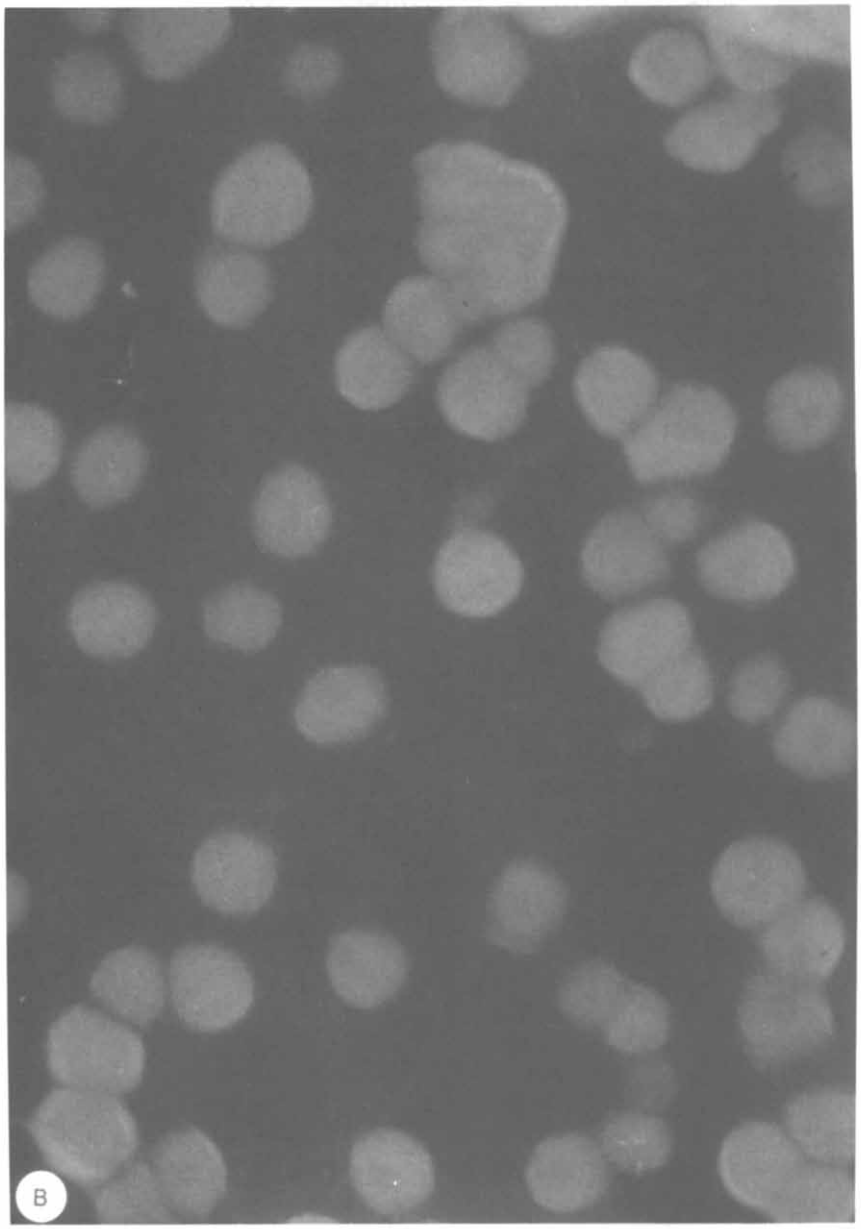

Fig. 4 (continued).

the marker enzyme for the basolateral plasma membrane. The contamination by microsomal proteins was estimated by measurement of glucose-6-phosphate activity. This enzyme was enriched 2-fold in the preparations. For photoaffinity labeling 2 mg of plasma membrane proteins were incubated for 2 min at 37°C in the presence of 20 μCi [^{3}H]bumetanide. Affinity labeling was performed with 3–6 flashes in a labeling device according to Frimmer and Ziegler [18].

Non-equilibrium pH gradient gel electrophoresis (NEPHGE) and electroelution of proteins

These methods were performed according to O'Farrell et al. [19], modified as described previously [20]. In brief, the gel rods consisted of 5.1 g urea, 2 ml 10% Triton X-100, 380 mg acrylamide, 20 mg bis-acrylamide, 0.5 ml ampholines (pH 3–10), 3.25 ml H_2O, 25 μl TEMED and 25 μl 10% ammonium peroxydisulfate. After centrifugation at 100 000 $\times g$ the proteins were directly resuspended in lysis buffer. Prefocusing was done at 500 V for 1 h followed by sample focusing at 2300 V for 4 h and at 1500 V for 2 h. The gel rods were then equilibrated in SDS-sample buffer and applied onto a preparative SDS slab gel. For isolation of the protein, Coomassie blue stained protein spots were excised from the gel and electroeluted in the presence of Laemmli buffer (pH 8.3) in a self made electroelution chamber for 3 to 5 h.

SDS slab gel electrophoresis

One-dimensional SDS gel electrophoresis was performed in 10% acrylamide/0.28% bis-acrylamide gels according to Laemmli [21]. Further details were described by Honscha et al. [20].

Western blotting

Protein samples which were separated by SDS gel electrophoresis were transferred by electroblotting to nitrocellulose [22]. After washing in TBS (10 mM Tris-HCl (pH 8.0), 150 mM NaCl) the blot was incubated for at least 1 h in TBS with 0.05% Tween 20 (TBST). The dilution of the antibody against the PDI was 1:5000. After incubation for 2 h the blots were briefly washed and then incubated with an alkaline phosphatase conjugated goat anti-rabbit antibody. The blots were stained by nitroblue tetrazolium and 4-bromo-3-chloroindolyl phosphate.

Preparation of polyclonal antibodies

Male New Zealand rabbits were immunized with the recombinant fusion protein. Three cycles of subcutaneous injections with 50 μg of the fusion protein were done. For the first immunization the antigens were mixed with Freund's complete adjuvant and later Freund's incomplete adjuvant was used.

Serum preparation

The serum was prepared from the blood of the animals according to the method of Harlow and Lane [23], and stored in aliquots at $-20°$ C.

Immunofluorescence

Fresh isolated hepatocytes were fixed with 3% freshly prepared paraformaldehyde in 100 mM phosphate-buffered saline (PBS) (pH 7.4) for 1 h at 4°C. No additional membrane permeabilizing agents were used in order to detect only the plasma membrane bound fractions of the proteins. The cells were grown on 12 mm cover slips. After washing with PBS (pH 7.4) (three times for 10 min each) the cells were incubated with the antiserum at a dilution of 1:200 for 18 h at 4°C. The cells were washed again, then incubated with fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG (1:200 diluted, 2 h at room temperature in the dark) and mounted onto slides with a glycerol-based medium containing *p*-phenylenediamine in order to reduce the photobleaching of the fluorescein [24]. The hepatocytes were visualized with a Zeiss Universal microscope, equipped with appropriate fluorescence filters. For control, the hepatocytes were incubated with pre-immune serum.

Results

PDI is a known soluble and abundant protein which is mainly located in the endoplasmic reticulum [1]. The putative involvement in hepatocellular transport processes requires that this enzyme is also located in the plasma membrane of hepatocytes.

To test this hypothesis we constructed a bacterial expression vector (pEX-PDI 324) which produces large amounts of a recombinant β-galactosidase-PDI fusion protein after induction. The fusion protein was cloned by excising a small fragment (324 bp) of the coding region of the published PDI-cDNA clone [11] and by inserting this fragment behind the 3′ end of the β-galactosidase gene. The resulting clone which was named pEX-PDI 324 produced the desired β-galactosidase-PDI fusion protein. From 2.5 ml bacterial culture the recombinant fusion protein was prepared and checked by Western blotting (Fig. 1, lanes 2–4) with an

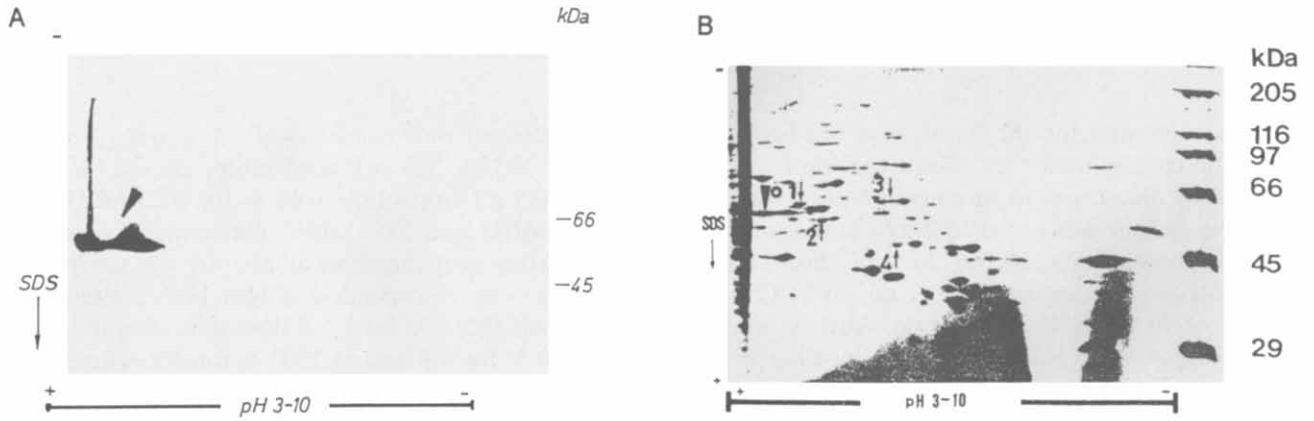

Fig. 5. Identification of the location of PDI in a two-dimensional gel with anti-PDI antibody. 1 mg of sinusoidal plasma membranes were separated by two-dimensional gel electrophoresis. The proteins were then transferred to nitrocellulose by electroblotting (see Materials and Methods). The nitrocellulose sheet was then incubated with anti-PDI antibody at a dilution of 1:4000. The PDI is marked by an arrow. Fig. 5B shows a Coomassie blue staining of a corresponding two-dimensional gel. The previously identified bumetanide binding proteins [20] are numbered according to the cited paper. The location of PDI is indicated by a circle.

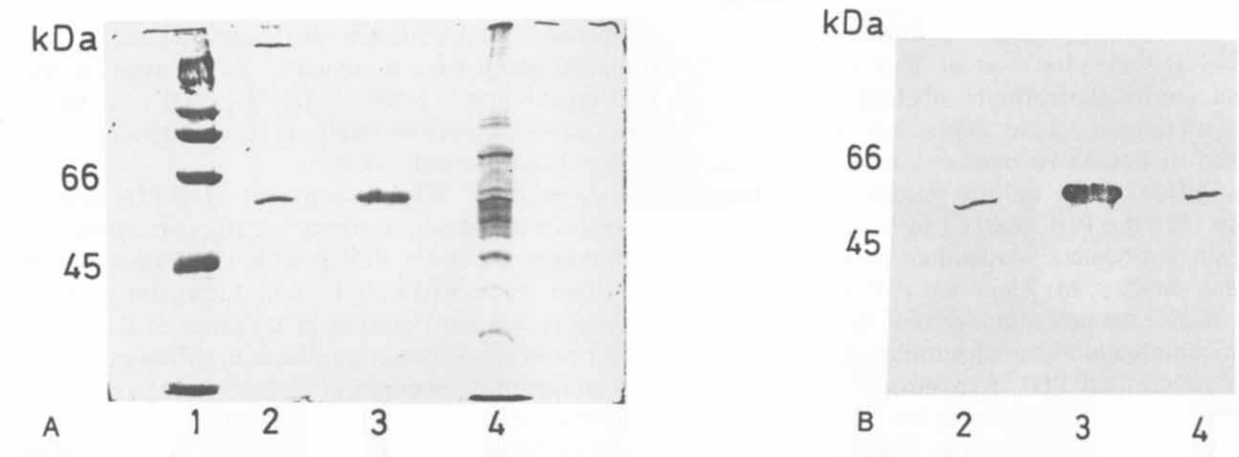

Fig. 6. Electroelution of the identified PDI-protein from two-dimensional gels and Western blotting. Fig. 6A shows a Coomassie blue-stained SDS gel with the electroeluted protein, which had been identified by anti-PDI antibody in two-dimensional gels in comparison with the purified PDI-protein. The PDI-protein was isolated from 20 preparative two-dimensional gels and electroeluted. Details of the procedure are described in the Materials and Methods section and by Honscha et al. [20]. Lane 1: 5 μg molecular mass marker proteins with molecular masses of 29 kDa, 45 kDa, 66 kDa, 97 kDa and 116 kDa. Lane 2: 100 ng of the purified PDI-protein. Lane 3: 500 ng of the electroeluted protein. Lane 4: 20 μg sinusoidal plasma membrane protein. Fig. 6B shows the immunostaining of the gel with anti-PDI antibodies.

antibody against the T_3 binding protein [25]. Based on the apparent identicalness of PDI and the thyroid binding protein [1,25] this T_3-antibody recognized the PDI fusion protein (Fig. 1, lane 3). This antibody also recognized purified T_3 binding protein (lane 2) and purified microsomal PDI-protein from bovine liver (lane 4). The PDI fusion protein was then prepared on a preparative scale (500 ml bacterial culture volume) and after electroelution the protein was used for the immunization of rabbits. The titer and the specificity the resulting anti-PDI serum was checked by Western blotting (Fig. 1, lane 5–7). Microsomal PDI which was used as a control of the immunoblots was isolated from bovine liver. Table I shows the enrichment of the specific activity of PDI during purification from bovine liver. A 295-fold enrichment of the microsomal PDI was achieved. Fig. 2 shows an SDS gel of aliquots of the protein from the different purification steps. Fig. 3 shows the specific activity of the isolated PDI as determined by the ability to degrade insulin.

With the anti-PDI serum the location of the PDI in intact hepatocytes was determined by direct immunofluorescence (Fig. 4). In comparison with the control, in which pre-immune serum was used (Fig. 4B), the presence of a PDI-form in the plasma membrane of hepatocytes was verified (Fig. 4A). The labeling of the cells revealed clusters of the antigen which formed spots of immunofluorescence on the surface of the cells. These data clearly shows that a part of PDI is located in the plasma membrane of hepatocytes.

The antigen was also present in purified sinusoidal plasma membranes from rat liver. Fig. 5A shows an immunoblot of a two-dimensional gel with 1.5 mg sinusoidal plasma membranes. The immunosignal reveals two spots of 57 kDa proteins with a thin line between them at an apparent p*I* of 4.5. Fig. 5B shows a corresponding two-dimensional gel after Coomassie blue staining.

A membrane-form of PDI was supported to be involved in the hepatic uptake of bile acids because the protein bound photoactivated bile salt affinity labels [6,7]. Hepatic uptake of bile acids is closely related to the uptake of the organic anion bumetanide [10,26] and comparable membrane proteins were labeled by pho-

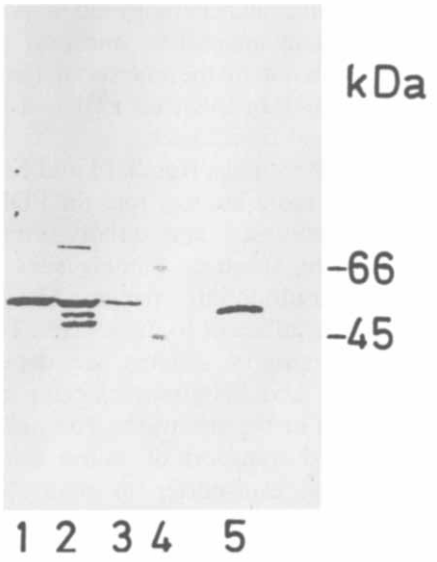

Fig. 7. Western blot of protein samples from different cell lines. 20 μg of cellular protein from each cell line was separated by SDS gel electrophoresis and then transferred to nitrocellulose. The blot was then incubated with the anti-PDI antibody. Lane 1: hepatocytes. Lane 2: AS-30D. Lane 3: FAO. Lane 4: marker proteins with molecular masses of 45 kDa and 66 kDa. Lane 5: 100 ng of purified PDI-protein.

toactivated bumetanide [16]. In a previous paper [20] we described the identification of four bumetanide binding proteins by photoaffinity labeling and subsequent two-dimensional gel electrophoresis. These proteins, marked in Fig. 5b by numbers, have molecular masses of 45 kDa, 52 kDa and two proteins of 60 kDa. It is obvious that the PDI, marked by a circle, is not among the labeled bumetanide binding proteins.

For further analysis, the identified PDI was excised from two-dimensional gels and electroeluted. Fig. 6A, lane 3 shows an aliquot of the electroeluted protein in comparison to purified PDI from bovine liver. Both proteins have identical mobilities in the SDS gel. Fig. 6B shows by Western blot analysis that the protein from sinusoidal plasma membranes which was identified in two-dimensional gels by the anti-PDI antibody and then excised from the gels is immunologically identical to the isolated microsomal PDI-protein from bovine liver.

Our results clearly demonstrate that the PDI is not among the binding proteins for the organic anion bumetanide and this makes it very unlikely that PDI is involved in the hepatocellular uptake of this compound. This statement is supported by the fact that transport-deficient cell lines (AS 30D, FAO-cells) also contain PDI as revealed by Western blotting (Fig. 7, lane 2 and 3). In comparison to hepatocytes (Fig. 7, lane 1), the AS-30D cells (lane 2) contain nearly equal amounts of PDI.

Discussion

Several lines of evidence indicated a possible involvement of PDI in membrane transport processes: First, PDI is a subunit of the microsomal triglyceride transfer protein [3,4]. In this case PDI is a functional part of a microsomal transporter.

Second, in earlier studies Robillard and Konings [5], have postulated a more indirect role for PDI in membrane transport processes. The authors proposed that the affinities of the substrate binding sites are regulated by dithiol–disulfide interchanges. These induced changes should be sufficient to drive active transport.

Third, many transport systems are dependent of sulfhydryl groups, and SH-group blocking agents inhibit the transport of the substrates. Examples are the system A-mediated transport of amino acids [27,28], the organic cation transporter in renal basolateral membranes [29], the transporter for β-lactam antibiotics in the small intestine [30] and organic anion transporters in the liver. In this respect, hepatocellular uptake of sulfobromophthalein [31,32], bile acids [8] and of bumetanide [9] depend on functional membrane SH-groups.

Fourth, by photoaffinity labeling of rat liver plasma membranes, the photolabile bile acids 7,7′-azotaurocholate and azidobenzamidotaurocholate labeled proteins, which were assumed to be involved in bile acid uptake into hepatocytes [33–35]. PDI was among these labeled proteins (Refs. 6, 7, and Kramer, W., personal communication).

Two possibilities for an involvement of PDI in hepatocellular membrane transport of organic anions are considered: either the PDI itself is the transporter or the PDI is associated with the transducing unit and has regulatory functions exerted by a change of the transporter protein conformation due to disulfide exchange. By immunofluorescence of intact hepatocytes we demonstrate here that PDI is located indeed as a transmembrane protein in the plasma membrane of hepatocytes. These results are supported by the previous data from Agaki et al. [36,37] who analysed immunochemically the subcellular distribution of the PDI on ultra-thin sections in liver and pancreas. PDI was present in the ER and in the nuclear envelope of hepatocytes. The plasma membranes of exocrine pancreatic cells contained 20-fold more PDI than the plasma membrane of hepatocytes.

Our specific antibody which was generated from a recombinant fusion protein recognized PDI on intact cells, indicating that the protein extends out of the membrane. This may explain why photoactivated bile acids bound to this protein. On the other hand, PDI is clearly not among those proteins labeled by photoactivated bumetanide. We therefore conclude that PDI in its membrane form is not essential for bumetanide uptake in rat hepatocytes.

Acknowledgements

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