Isolation and complete covalent structure of liver microsomal paraoxonase

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Paraoxonase (PON1) is a serum esterase exclusively associated with high-density lipoproteins; it might confer protection against coronary artery disease by destroying pro-inflammatory oxidized lipids in oxidized low-density lipoproteins. Here I show that rabbit liver microsomes contain a PON analogue (MsPON) and report the isolation and complete covalent structure of MsPON. In detergent-solubilized microsomes, MsPON co-purifies with the microsomal triacylglycerol transfer protein (MTP) complex. MsPON was separated from the complex and purified to homogeneity under non-denaturing conditions. Automated sequence analysis of intact MsPON and peptides obtained from enzymic and chemical cleavages led to the elucidation of the complete covalent structure of MsPON. The protein is a single polypeptide consisting of 350 residues. The sequence of rabbit liver microsomal MsPON is 60% identical with that of rabbit serum PON1, and 84% identical with the sequence predicted by a human cDNA of unknown function, designated PON3. MsPON has a hydrophobic segment at the N-terminus

that might serve to anchor the protein to the microsomal membrane or to the MTP complex. Unlike in the serum enzyme, two potential N-glycan acceptor sites in MsPON are not glycosylated. An absence of N-glycans was also indicated in the rabbit liver MTP. MsPON has a single free cysteine residue at position 38 and a disulphide bond between Cys-279 and Cys-348. The microsomal enzyme lacks three residues at the N-terminus that are present in the serum protein. MsPON lacks four residues at the C-terminus that are present in the rabbit serum protein but absent from human serum PON1. On the basis of the observation that MsPON displays a high degree of similarity with serum PON1, it is proposed that MsPON might have a function related to that of PON1 in serum high-density lipoprotein complexes.

Key words: lipoprotein oxidation, high-density lipoproteins, microsomal triacylglycerol transfer protein, microsomal membrane proteins.

INTRODUCTION

The isolation and the amino acid sequences of a number of microsomal membrane esterases have been reported [1–4]. During these studies a ubiquitous 40 kDa protein co-purifying with the microsomal triacylglycerol transfer protein (MTP) was identified. Liver microsomal MTP, a heterodimer of 97 and 58 kDa polypeptides, transports lipids and is involved in the post-translational assembly of triglyceride-rich plasma proteins, very-low-density lipoproteins and chylomicrons (reviewed in [5]). Initial structural studies indicated that the 40 kDa protein is related to the serum esterases designated PON1. Serum paraoxonase (PON1) is an esterase in the plasma involved in the detoxification of organophosphate insecticides such as paraoxon. Initial studies by Mackness et al. [6] and more recent studies [7–11] demonstrated that serum PON contributes to the antioxidant protection conferred by high-density lipoproteins on low-density-lipoprotein oxidation. In the serum, PON is tenaciously bound to the high-density lipoproteins [6–8,12]. PON1 has been extensively characterized from rabbit and human serum [13–16]. Several laboratories have reported the presence of PON1 activity in liver microsomes [17–19]. Recently the isolation of a rat liver microsomal protein with esterase activity similar to the serum PON1 was reported, but owing to the limited sequence information presented, a relationship between the liver and the serum proteins could not be established [19]. A physiological role for microsomal PON has not been defined. It remains to be determined whether the rabbit liver microsomal PON analogue (MsPON) has a role in microsomal lipid peroxidation or in the function of MTP. As the first step in defining the functional role

of the MsPON protein, the isolation and determination of the complete amino acid sequence of rabbit liver microsomal MsPON is presented here.

EXPERIMENTAL

Materials

Detergents, enzyme substrates, cofactors, chromatographic media and chemicals were obtained from Sigma unless stated otherwise. Hydroxyapatite (HA-Ultragel) was a product of IBF Biotech (Villeneuve-la-Garenne, France). DEAE-cellulose (DE-52) was from Whatman. The Bio-Pro Q column was from Bio-Rad. Trypsin was obtained from Worthington. Endopeptidases Asp-N, Glu-C and endo-*N*-acetylglucosaminidase F (endo F) were from Boehringer Mannheim. *Achromobacter* peptidase Lys-C was obtained from Biochemical Diagnostics (Edgewood, NY, U.S.A.). CNBr was obtained from Pierce Chemicals. Solvents for HPLC and gel filtrations were from Burdick & Jackson. Molecular mass standards for SDS/PAGE were from Bio-Rad. The 66 kDa marker, BSA in the premixed Bio-Rad 'Low Range' standard set, frequently precipitated during storage and was therefore not present in the SDS/PAGE lanes of some standards. ¹⁴C-labelled molecular mass markers were from Sigma. Centricon 30 concentrators were obtained from Amicon.

Isolation of 40 kDa protein

Liver microsomes were isolated from New Zealand male rabbits and solubilized as described previously [20]. Microsomes were

Abbreviations used: DTT, dithiothreitol; endo F, endo-*N*-acetylglucosaminidase F; ER, endoplasmic reticulum; MsPON, rabbit liver microsomal PON analogue; MTP, microsomal triacylglycerol transfer protein; PDI, protein disulphide isomerase; PON, paraoxonase; PTH, phenylthiohydantoin.

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suspended in 10 mM potassium phosphate, pH 7.4, containing 20 % (v/v) glycerol, 0.1 mM dithiothreitol (DTT) and 1 mM EDTA, at a protein concentration of 15 mg/ml. Solubilization of microsomes was achieved by the addition of sodium cholate (1.2 mg/mg of protein). The solubilized material was fractionated with poly(ethylene glycol) as described in [20]. The 6–12 % (w/v) poly(ethylene glycol) precipitate was solubilized with Tergitol NP-10 (1.3 mg/mg of protein). The solubilized material was applied to a DEAE-cellulose column (250 ml) equilibrated with 5 mM potassium phosphate, pH 7.4, containing 20 % (v/v) glycerol, 0.1 % (v/v) Nonidet P40, 1 mM EDTA and 0.1 mM DTT (buffer A). All buffers used in the subsequent purification steps contained 0.1 % (v/v) Nonidet P40, 20 % (v/v) glycerol and 0.1 mM DTT (abbreviated as NPGD). After the sample had been loaded, the column was flushed with 2 column vol. of equilibrating buffer, followed by 500 ml of 10 mM potassium phosphate, pH 7.4, containing 25 mM NaCl and NPGD. The column was then developed with buffer A containing 125 mM NaCl. At this point, 6 ml fractions were collected at a flow rate of 25 ml/h. The 40 kDa protein was eluted between fractions 32 and 44 (see Figure 1A), or in a repeat experiment between fractions 42 and 52 (see Figure 1B). Aliquots of these fractions were dialysed against 10 mM potassium phosphate, pH 7.4, containing NPGD (buffer B), and applied to a second column of DEAE-cellulose (15 ml) equilibrated with buffer A. After the column had been washed with 100 ml of buffer B, the column was developed with a linear gradient of increasing concentration of NaCl (10–300 mM), 50 ml in each gradient vessel at 6 ml/h. Fractions containing MsPON from several preparations were dialysed and applied to a column of hydroxyapatite (75 ml) pre-equilibrated with 10 mM potassium phosphate, pH 7.4, containing 0.1 % (v/v) Nonidet P40, 100 μ M CaCl_2 and NPGD. The column was developed with equilibrating buffer; fractions containing 450 drops per tube were collected at 5 ml/h. At fractions 40, 55 and 70 the column was developed with a stepwise gradient of equilibrating buffer containing 50, 125 and 300 mM potassium phosphate, pH 7.4 (see Figure 2). Fractions 18–27 were pooled and concentrated on a Centricon 30; aliquots were injected on a 2 ml Bio-Pro Q column equilibrated with 20 mM Tris/HCl (pH 7.5)/0.1 % Nonidet P40/50 mM NaCl/20 % (v/v) glycerol. The column was washed with 20 ml of the equilibration buffer and developed with a 30 ml gradient of 50–500 mM NaCl at a flow rate of 1 ml/min.

Column fractions were monitored by SDS/PAGE with 4 % (w/v) polyacrylamide stacking gel and 8 %, 10 % or 12 % (w/v) polyacrylamide resolving gels (8 cm $\times$ 7 cm, 1.0 mm thick) with 10-lane or 15-lane gels. Electrophoresis was performed in the Bio-Rad minicell at room temperature for 40 min at 180 V. Large gels (14 cm $\times$ 16 cm, 1.5 mm thick) were first run at 40 mA for 2.5 h, then at 60 mA for 2.5 h. Gels were stained with 0.25 % Coomassie Blue in 40 % (v/v) methanol/10 % (v/v) acetic acid, and destained in 10 % (v/v) acetic acid/40 % (v/v) methanol. For micro-sequence analysis, protein bands were eluted from the large gels with 40 mM ammonium bicarbonate/0.02 % SDS buffer with the use of the Elutrap chamber from Schleicher & Schuell, in accordance with the manufacturer's instructions.

The esterase activity of the 40 kDa protein was determined with phenyl acetate as the substrate with the Ultraspec 3000 spectrophotometer. Aryl esterase activity was measured by using a molar difference absorption coefficient (difference between the absorption coefficients between phenol and phenyl acetate) of $1310 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 270 nm with 1.0 mM phenyl acetate in 50 mM Tris/HCl, pH 8.1, containing 1.0 mM CaCl_2 [16]. The specific activity of the purified preparations towards phenyl acetate was 5–10 nmol/min per mg.

For glycerol gradient centrifugation studies, samples were concentrated and dialysed on Centricon 30 systems with 10 mM Tris/acetate, pH 8.1, containing 130 mM NaCl, 2 % (v/v) glycerol and 0.1 % (v/v) Nonidet P40 (buffer C). The concentrated samples were reconstituted to the original volume with buffer C. This procedure was repeated to obtain a 2 % (v/v) glycerol concentration in the sample. Aliquots (2 ml) of dialysed purified enzyme were layered on 36 ml gradients [8–30 % (v/v) glycerol] in Beckman 38 ml centrifuge tubes. The glycerol gradient contained the sample dialysis buffer. The gradient-containing tubes were centrifuged for 16 h at $84000 g_{\text{max}}$, in a Beckman SW 28 rotor at 4 °C, after which 15 fractions of 2–2.5 ml were collected manually from top to bottom of each tube; these were then analysed by SDS/PAGE.

Sequence analysis

Protein/peptide hydrolysis for amino acid analysis was performed with 6 M HCl in the gas phase, at 150 °C for 1 h [21]. Reduction, carboxymethylation, succinylation, and enzymic and chemical cleavages were performed as described previously [22]. The initial purification of complex peptide mixtures involved gel filtration on a 1.5 cm $\times$ 100 cm column of LH60-Sephadex equilibrated with formic acid/ethanol (3:7, v/v) as the solvent. Peptide mixtures in gel-filtration fractions were resolved by reverse-phase HPLC. The latter methodology has been described in detail previously [22]. Reverse-phase columns employed for HPLC separations included C_4 (Vydac, Hesperia, CA, U.S.A.) (4 cm $\times$ 0.46 cm or 15 cm $\times$ 0.46 cm) and Waters C_{18} Bondapac (30 cm $\times$ 0.39 cm). Peptide mixtures were dissolved in 88 % (v/v) formic acid before injection on the column. Solvent conditions for the reverse-phase columns were as follows. Solvent A was 0.1 % trifluoroacetic acid; solvent B was 0.1 % trifluoroacetic acid in 75 % (v/v) acetonitrile. A linear gradient of 0–100 % solvent B in solvent A was applied at a flow rate of 1.0 ml/min. Sequence analysis was performed on an Applied Biosystems model 470A sequencer equipped with a model 120A phenylthiohydantoin (PTH) analyser, in accordance with the manufacturer's instructions. Solid-phase sequencing was performed on a 6600 ProSequencer system (MilliGen/Bioscience, Novato, CA, U.S.A.) as described previously [22].

Specific labelling of the cysteine residues was as follows. A 40 kDa protein preparation was dialysed against 100 vol. of buffer (degassed by bubbling with nitrogen) containing 20 mM Tris acetate, pH 8.1, 20 % (v/v) glycerol, 0.1 % (v/v) Nonidet P40 and 1 mM EDTA. To 5 nmol of 40 kDa protein in 5 ml of dialysis buffer, 7 ml of neutralized 8 M guanidinium chloride solution was added, followed by 50 μ Ci of [^{14}C]iodoacetamide (specific radioactivity 55 mCi/mmol). After 100 min at room temperature, 50 μ l of 1 M iodoacetamide was added and the reaction mixture was incubated for 1 h at 37 °C. The reaction mixture was then reduced by the addition of 125 μ l of 1 M DTT. After 1 h at 37 °C, 350 μ l of 1 M iodoacetic acid was added and the alkylation continued for 1 h at 37 °C. After dialysis against 5 mM ammonium bicarbonate containing 0.1 % (v/v) 2-mercaptoethanol, the preparation was freeze-dried and subjected to hydrolysis with CNBr or Lys-C protease. The enzymic digests were first resolved on a column of LH60-Sephadex or directly by HPLC. Aliquots of peptide-containing material were withdrawn for measurement of the ^{14}C content with a Packard scintillation counter. The remaining peptide fraction was used for the sequence analysis. Carboxymethyl- and carboxyamidomethyl-PTH-cysteine derivatives were distinguished by their characteristic elution positions on the PTH analyser.

Enzymic deglycosylation of MsPON and MTP preparations

Figure 1 SDS/PAGE analysis of DEAE-cellulose column fractions containing MsPON and MTP proteins

These fractions were obtained by eluting the column with 125 mM NaCl as described in the text. The number above each lane denotes the fraction number of the sample. **(A)** SDS/PAGE [12% (w/v) gel]: a 15-lane gel was stained with Coomassie Blue. The asterisks denote the 97 kDa MTP and 40 kDa MsPON. The molecular masses of the markers in lane S are indicated (in kDa) at the left. **(B)** Repeat of the experiment described in **(A)** using a different batch of solubilized rabbit-liver microsomes. **(C)** SDS/PAGE [12% (w/v) gel] of fraction 42 **(B)** stained with Coomassie Blue. Protein bands identified by sequence analysis were assigned a number as shown at the right of the gel. The N-terminal sequences of proteins identified are given in Table 1.

with endo F was performed on samples concentrated on C-30 concentrators and the parent buffer was exchanged with 20 mM sodium phosphate, pH 6, containing 1% (v/v) 2-mercapto-

Figure 2 SDS/PAGE analysis of MsPON and MTP in fractions eluted from a hydroxyapatite column

Dialysed MsPON- and MTP-containing fractions from a second DEAE-cellulose column chromatography step were applied to the column and developed with a linear gradient of potassium phosphate (10–300 mM), as described in the text. **(A)** SDS/PAGE [12% (w/v) gel], stained with Coomassie Blue, of fractions that were eluted at approx. 60 mM potassium phosphate. Lane PC, precolumn sample. **(B)** SDS/PAGE [10% (w/v) gel] of fractions that were eluted at high potassium phosphate concentration (more than 200 mM). The number above each lane denotes the fraction number of the sample. Lane S, purified MsPON. Asterisks denote MTP and PDI. The positions of molecular mass markers are indicated (in kDa) at the left of each panel.

ethanol and 0.2% SDS. The samples were incubated with 0.3 unit of endo F for 16 h at 37 °C. Samples of the digest were analysed by SDS/PAGE. The glycoprotein microsomal esterase 1 [1] was used as a positive control.

RESULTS

The purification procedure for detergent-solubilized MsPON is outlined in Figures 1–3. MsPON was substantially purified from detergent-solubilized microsomes on a column of DEAE-cellu-

Table 1 N-terminal sequence analysis of proteins present in fraction 42 from the DEAE-cellulose column

Erp 57 is an ER protein that is similar to rat liver ER-60.

Band in Figure 1(C)	Approx. molecular mass (kDa)	Sequence	Protein
1	97	VKLGSTGLSLNNDRL	MTP
2	57	SDVLELTPDNFE	Erp 57
3	57	DAPEEEDNVLVKSS	PDI
4	50	MDLLIILGICLSCVV	Cytochrome P-450
5	47	MLLELLLASVLGFVI	Epoxide hydrolase
6	40	LLLTLLFASLAFVGE	MsPON
7	31	LHTKGALPLDVTIFY	Unknown protein
8	17	Acetyl-AAQSDKDKVYYTLEE	Cytochrome <i>b</i> ₅

Figure 3 SDS/PAGE analysis of purified MsPON

The MsPON was isolated by chromatography on DEAE-cellulose, hydroxyapatite and Bio-Pro Q columns. The SDS/PAGE was on a 10% (w/v) gel stained with Coomassie Blue. Lane 1, molecular size markers; lanes 2 and 5, MsPON preparation after first DEAE-cellulose column step; lanes 3 and 4, purified MsPON. The molecular masses of the markers are indicated (in kDa) at the left.

Figure 4 Evidence for the absence of N-glycans from MsPON and from the 97 and 58 kDa subunits of the MTP complex

SDS/PAGE [10% (w/v) gel], stained with Coomassie Blue, of MsPON and MTP preparations incubated in the absence (—) and presence (+) of endo F (lanes 2 and 3, and 4 and 5 respectively). Esterase 1 was used as positive control (lanes 6 and 7). The positions of molecular mass markers are indicated (in kDa) at the left.

lose. A stepwise gradient of increasing NaCl concentration eluted MsPON at approx. 140 mM NaCl. The reproducibility of these steps is shown in Figure 1(B). Here a different batch of liver microsomes was solubilized and then resolved on a DEAE-cellulose column. Even though the quantity of microsomes solubilized and applied to the column shown in Figure 1(B) was approx. 30% less owing to the differences in liver weight of the animals, all of the major protein bands present in Figure 1(A) can be identified in Figure 1(B). Major proteins that were co-eluted with MsPON included cytochrome b_5 , MTP, protein disulphide isomerase (PDI) and an endoplasmic reticulum (ER) protein, Erp 57, that is similar to rat liver ER-60 (Figure 1C and Table 1). Minor proteins eluting from the DEAE-cellulose column at approx. 140 mM NaCl included epoxide hydrolase and a 30 kDa protein with a KEEL C-terminus. Except for the epoxide hydrolase, the proteins that were eluted on development of the column with 125 mM NaCl were not present in the void volume of the column or in fractions eluted with the 25 mM NaCl step. Although the major band of epoxide hydrolase was eluted with 25 mM NaCl, small quantities of this protein continued to be eluted at higher NaCl concentration. Proteins present in MsPON-containing fractions were identified by sequence analysis of their N-termini (Table 1).

Figure 5 SDS/PAGE of glycerol-density-gradient fractions after staining with Coomassie Blue

A 2.5 ml aliquot of partly purified MsPON preparation obtained from the second DEAE-cellulose column shown in lane PC was layered on a glycerol gradient and fractionated by centrifugation as described in the Experimental section. Fractions 1–15 are from the top to the bottom of the gradient. The bands representing MsPON, PDI and MTP are indicated by asterisks. The positions of molecular mass markers are indicated (in kDa) at the left.

Chromatography of MsPON fractions on a second DEAE-cellulose column, followed by further purification on a hydroxyapatite column, yielded MsPON–MTP complexes in addition to MsPON fractions that were free from MTP and PDI proteins (Figure 2). Further purification of MsPON fractions by anion-exchange chromatography on a Bio-Pro Q column gave a preparation that on SDS/PAGE gave a single band with a mobility of approx. 40 kDa (Figure 3). The purified MsPON hydrolysed phenyl acetate in the presence or absence of Ca^{2+} . The aryl esterase activity in MsPON preparations was not inhibited by supplementation with di-isopropylphosphofluoridate.

The carbohydrate status of the enzyme was examined by deglycosylation with endo F followed by SDS/PAGE (Figure 4). Incubation of the MsPON and MTP with endo F did not decrease their apparent molecular masses. To confirm that MsPON interacted with the MTP, partly purified preparations were subjected to sucrose- or glycerol-density-gradient centrifugation. As seen in Figure 5, when partly purified MsPON preparations were fractionated by glycerol-density-gradient centrifugation, MsPON, MTP and PDI were recovered from the mid- and late portions of the 8–30% glycerol gradient.

The amino acid sequence determination of MsPON involved the reduction and carboxymethylation of the protein, followed by chemical and enzymic cleavages. Resolution of the digests was initially accomplished by gel filtration on an LH-60 Sephadex column followed by HPLC of the individual fractions. The resolution of the CNBr and Lys-C digests by the LH-60 Sephadex column is seen in Figures 6(A) and 6(B). The complete amino acid sequence and the order of all peptides obtained by the primary cleavages were determined by isolation and sequence analysis of peptides subcleaved with Asp-N endoproteases, as shown in Figure 6(C). Solid-phase and gas-phase Edman degradation identified all residues in the peptides, including the two potential N-glycosylation sites at Asn-46 and Asn-265. Unmodified asparagine residues were identified at these positions.

To identify the state of the three cysteine residues present in MsPON, the protein was denatured with guanidinium chloride and treated with iodoacetamide or [^{14}C]iodoacetamide followed by reduction and alkylation with iodoacetic acid. This carboxymethylation protocol was designed to label free (reduced) cysteine residues with [^{14}C]iodoacetamide and disulphide-linked (oxidized) cysteine residues with iodoacetic acid. The modified protein was fragmented and the resulting digest was resolved by HPLC.

Figure 6 Gel filtration of MsPON digests on a column of LH-60 Sephadex (1.5 cm × 100 cm) equilibrated with formic acid/ethanol (3:7, v/v)

(A, B) Effluent fractions were collected at a flow rate of 10 ml/h. Bars indicate the fractions of a particular peptide pooled for HPLC, and the number below the bar indicates the number of residues in the peptide. (A) CNBr digest of approx. 4 nmol of protein; (B) gel filtration of the Lys-C endoprotease digest of 3 nmol of protein. (C) Complete amino acid sequence of rabbit liver microsomal MsPON. Peptides obtained by CNBr and Lys-C endoprotease digestion are designated with the prefixes CB and K respectively. Subcleavage of peptides by trypsin or endoprotease Asp-N are designated by the prefixes T and D respectively. A disulphide bond links Cys-279 to Cys-348.

No radioactivity was present in peptides corresponding to segments containing Cys-279 and Cys-348. Radioactivity was confined to the peptide containing Cys-38; therefore a disulphide bond between Cys-279 and Cys-348 was assigned.

DISCUSSION

The amino acid sequence of MsPON is summarized in Figure 6(C). The polypeptide consists of 350 residues with a calculated molecular mass of 39049 Da. Peptides obtained from digests with CNBr and Lys-C endoprotease were characterized extensively. The mature MsPON has retained an uncleaved signal

sequence that might function as a binding segment to membrane or to MTP. Sequencer analysis of peptides containing the potential Asn-Xaa-Ser/Thr glycosylation sites, at positions 46 and 265, displayed unmodified asparagine derivatives, showing that the protein is not N-glycosylated at those sites. An absence of N-glycans was also indicated by digestion with endo F (Figure 4).

MsPON has one disulphide bridge between Cys-279 and Cys-348, which would favour a luminal orientation of the protein despite the absence of N-glycans. Disulphide bonds in membrane proteins are usually found in segments exposed towards the luminal side of the ER membrane. It is uncommon to find

Figure 7 Comparison of PON1 and PON3 sequences

(A) Alignment of the amino acid sequence of rabbit microsomal MsPON with the deduced sequence of rabbit serum PON1 cDNA. The top sequence shows the serum protein [14,16]. Amino acid residues that are identical in both proteins are denoted by asterisks. In the mature serum protein the N-terminal methionine residue is deleted [14]; a disulphide bond is assigned between Cys-42 and Cys-353 [23]. (B) Comparison of amino acid sequence of MsPON with the two known deduced sequences of PON3 cDNA [24]. MsPON is denoted by R, and PON3 sequences of mouse and human are denoted by M and H respectively. Amino acid residues that are identical in all proteins are denoted by asterisks.

disulphide bonds in polypeptide segments oriented towards the cytosolic side of the membrane. Further study is needed to determine the exact orientation of MsPON in the membrane.

A comparison of the rabbit serum and the rabbit liver microsomal proteins is shown in Figure 7(A). The two proteins share approx. 60% identical residues. MsPON has three residues deleted from the N-terminus and four residues deleted from the C-terminus that are present in the serum protein. The human serum protein also shows an absence of four residues at the C-terminus. In both mature proteins (MsPON and PON1) the N-terminal hydrophobic signal-peptide-like sequence is retained. The observation that an unclipped signal peptide is present in mature PON1, a protein destined for secretion, is to my knowledge unprecedented. Nevertheless the serum protein undergoes N-glycosylation typical of secreted proteins [16]. The signal sequence in the serum protein has been implicated in the interaction with the high-density lipoprotein particle with which it is exclusively integrated [12]. Detergents are required to dissociate PON1 from apolipoprotein A1 [12]. An internal stretch of hydrophobic residues (196–211) present in PON1 is interrupted by several polar residues in MsPON.

The rabbit serum PON1 contains four potential N-glycosylation sites, but only Asn-253 and Asn-324 were found to be glycosylated [16]. In MsPON two potential N-glycan sites, Asn-46 and Asn-265, although conserved in PON1, were not glycosylated. Interestingly, Asn-265, corresponding to Asn-270 in rabbit serum PON1, was not glycosylated, as shown by direct sequencing [16].

A disulphide bond between Cys-42 and Cys-353 has been assigned in PON1 [23]. In MsPON a free cysteine is placed at position 38 and a disulphide bond assigned between Cys-279 and Cys-348. In PON1 Cys-283 has been implicated in the catalytic site of the enzyme, on the basis of conservation of this site in serum PONs of all species investigated, and because PON1 esterase activity could be inhibited by organic mercurial compounds reacting with free thiol groups [23]. It has, however, been reported that replacement of Cys-283 by a serine or alanine residue through site-directed mutagenesis failed to alter the PON enzymic activities of the mutant cells [23]. Of the 15 tyrosine residues present in the serum protein 10 are conserved; 6 of the 10 histidine residues are retained in MsPON. The active site of the PON1 enzyme has not been identified, and until recently the

physiological substrate was unknown. The recent implication of PON1 in inhibiting the oxidation of low-density lipoprotein [9–11] provides a new dimension for the assignment of the physiological role of PON1.

The human genome contains two additional *PON1*-like genes, designated *PON2* and *PON3*, linked to the *PON1* gene on chromosome 7 [24]. The protein products of the *PON2* and *PON3* genes have not been isolated. As seen in Figure 7(B), MsPON is 84% and 76% identical with the deduced cDNA sequences of human and mouse *PON3* genes, implying that the PON equivalent in the rabbit liver microsomes is PON3. The human PON3 lacks nine residues present at the N-terminus of rabbit and mouse PON3. The absence of these residues from human protein is most probably due to incomplete sequence data for the human PON3. The degree of identity between human PON1 and human PON3 is 60% [24], which is very similar to that between rabbit PON1 and MsPON. A comparison of human PON3 with human PON2 displays 70% identity [24], and MsPON3 is 63% identical with human PON2.

The reports on the isolation of a PON-like enzyme from mouse rat liver microsomes by two independent groups is of interest [17–19]. In the paper by Rodrigo et al. [19] the enzyme (RatLP) was characterized with respect to its N-terminal sequence analysis and two internal sequences. The N-terminus of RatLP has three additional residues, absent from the MsPON and the mouse PON3 but present in all serum PONs. The isolation of MsPON and RatLP involves an initial DEAE-cellulose column chromatography step; both MsPON and RatLP proteins seem to be eluted by approx. 150 mM NaCl. The apparent molecular masses of MsPON and RatLP seem to be identical. Furthermore, as seen in Figure 1, there is only one protein band with a molecular mass of 37–43 kDa. Taken together, these points would imply that the liver microsomal preparations designated RatLP represent the same isoform as MsPON. At present, more complete amino acid sequences for any of the PON proteins from rat species are not available.

The finding that MsPON interacts with the microsomal MTP is of interest in the assignment of the physiological role of MsPON in liver microsomes. The microsomal MTP is a heterodimer composed of a unique 97 kDa unit and the 58 kDa protein PDI. The MTP heterodimer is essential for the assembly of very-low-density lipoproteins and chylomicrons [25]. Mutations in MTP result in an absence of apolipoprotein-B-containing lipoproteins in the plasma of abetalipoproteinaemic individuals [26]. The detailed mechanism and the number of components involved in the MTP-dependent secretion of lipoproteins remain to be elucidated.

The 97 kDa subunit in MsPON preparations was identified by N-terminal and internal peptide sequence analyses (Table 1). Although the N-terminal sequence of the mature 97 kDa subunit of the MTP protein has not been reported, the mature rabbit protein starts with residue 19 at the predicted cleavage site of the signal peptidase in the human protein [26]. The 15 N-terminal residues in the rabbit 97 kDa subunit differ from the human protein only at positions 21–23. PDI is a ubiquitous protein with multiple functions localized in the lumen of the ER [27]. Its disulphide isomerase activity is used to pair disulphide bonds properly in newly synthesized proteins. PDI also has a chaperone activity that is independent of its disulphide isomerase activity [28]. The importance of PDI to the MTP complex was illustrated in experiments expressing the 97 kDa unit alone and with the PDI in insect Sf9 cells. When Sf9 cells were infected with viruses expressing the 97 kDa unit alone, MTP activity was not detectable and the 97 kDa protein was found as an insoluble aggregate [29]. In contrast, when Sf9 cells were co-infected with

viruses expressing PDI and the 97 kDa subunit, MTP activity was detectable, and both PDI and the 97 kDa unit were found in the soluble fraction. Despite its luminal localization, the 97 kDa protein in rabbit liver microsomes is not N-glycosylated. The amino acid sequence of the human 97 kDa subunit does not contain the luminal retention signal KDEL or a dibasic ER retrieval motif. The 97 kDa subunit also lacks transmembrane domains. Presumably, PDI, in addition to maintaining the soluble state of the 97 kDa subunit, also functions to retain the subunit in the lumen of the ER.

During development of the isolation procedure it was evident that MTP and PDI co-purified with MsPON. To confirm the MsPON–MTP interaction, glycerol gradient centrifugation was employed. In a previous study, I and others used this method to show that microsomal oligosaccharyl transferase is a protein complex composed of 66, 63 and 48 kDa subunits [30,31]. SDS/PAGE analysis of fractions obtained by glycerol-density-gradient centrifugation of partly purified MsPON/MTP is shown in Figure 5. The three polypeptides that migrated into the glycerol gradient designated by arrows corresponded to the 97 kDa subunit, PDI and MsPON.

In summary, I have purified and determined the complete covalent structure of a ubiquitous liver microsomal protein that displays a high degree of similarity with the serum esterase *PON1* and approx. 80% identity with the human *PON3* gene product. I have shown that this protein co-purifies with MTP, implying a role in the MTP-dependent lipid transfer and assembly of very-low-density lipoproteins. This is the first report on the isolation, determination of the covalent structure and localization of the *PON3* gene product in the liver ER. The role of MsPON in lipid transport, lipoprotein assembly and lipid peroxide hydrolysis is a subject for future studies.

I thank George Korza for his outstanding technical assistance, and Dr. F.S. Heinemann (Department of Pathology, Hoag Memorial Hospital, Newport Beach, CA, U.S.A.) and Dr. Craig Gerard (Children's Hospital, Harvard Medical School, Boston, MA, U.S.A.) for many helpful discussions and critical reading of the manuscript. This work was supported by Grant R01 GM-26351 from the National Institutes of Health.

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Received 1 September 1998/4 November 1998; accepted 30 November 1998