Rabbit Serum Paraoxonase 3 (PON3) Is a High Density Lipoprotein-associated Lactonase and Protects Low Density Lipoprotein against Oxidation*

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The paraoxonase gene family contains at least three members: PON1, PON2, and PON3. The physiological roles of the corresponding gene products are still uncertain. Until recently, only the serum paraoxonase/arylesterase (PON1) had been purified and characterized. Here we report the purification, cloning, and characterization of rabbit serum PON3. PON3 is a 40-kDa protein associated with the high density lipoprotein fraction of serum. In contrast to PON1, PON3 has very limited arylesterase and no paraoxonase activities but rapidly hydrolyzes lactones such as statin prodrugs (e.g. lovastatin). These differences facilitated the complete separation of PON3 from PON1 during purification. PON3 hydrolyzes aromatic lactones and 5- or 6-member ring lactones with aliphatic substituents but not simple lactones or those with polar substituents. We cloned PON3 from total rabbit liver RNA and expressed it in mammalian 293T/17 cells. The recombinant PON3 has the same apparent molecular mass and substrate specificity as the enzyme purified from serum. Rabbit serum PON3 is more efficient than rabbit serum PON1 in protecting low density lipoprotein from copper-induced oxidation. This is the first report that identifies a second PON enzyme in mammalian serum and the first to describe an enzymatic activity for PON3.

The paraoxonase gene family in mammals includes at least three members: PON1, PON2 and PON3 (1). The PON genes appear to have arisen by gene duplication of a common evolutionary precursor because they share considerable structural homology and are located adjacently on chromosome 7 in humans and on chromosome 6 in mice (1). PON1, also known as serum paraoxonase/arylesterase (EC 3.1.8.1), is closely associated with high density lipoproteins (HDL)¹ and catalyzes the hydrolysis of a variety of aromatic carboxylic acid esters and several organophosphates (2, 3). Recently, we reported that purified human and rabbit serum PON1 also hydrolyze of a variety of lactones and cyclic carbonate esters, including naturally occurring lactones and pharmacological agents (4, 5). PON1 requires Ca²⁺ for both its stability and hydrolytic activity (2), and the latter is stimulated by several phospholipids (6). Any physiological role of PON1 is still speculative; however, several lines of evidence suggest that this enzyme protects against atherosclerosis by preventing the oxidation of low density lipoproteins (LDL) (see Refs. 7–9 for reviews). Oxidized phospholipids and/or their degradation/metabolic products may be physiological substrates of PON1 (10). PON1 knockout mice are more susceptible to organophosphate toxicity as well as to atherosclerosis produced by an atherogenic diet (11). PON1 also protects against bacterial endotoxin-induced toxicity (12).

The roles of PON2 and PON3 are much less well understood than are those for PON1. Within a given species, PON1, PON2, and PON3 share about 70% identity at the nucleotide level and about 60% identity at the amino acid level, whereas between the mammalian species particular PONs (1, 2, or 3) share 81–91% identity on the nucleotide level and 79–90% identity at the amino acid level (1, 12). To date, all PON2 and PON3 cDNAs sequenced lack the three nucleotide residues of codon 106 in PON1 (1, 12). Some lactone hydrolases previously described in bacteria (13) and in fungi (14) share remarkable structural homology with the PON family members, and we hypothesized that the lactonase activity rather than arylesterase or organophosphatase activities could be a common feature of the PON proteins. In the present study, a rabbit serum lactonase distinct from PON1 was purified and characterized. This is a 40-kDa protein that has an N-terminal sequence (25 amino acids) that exactly matches the deduced amino acid sequence predicted by the rabbit PON3 cDNA we have cloned and that agrees closely with the sequence reported recently for rabbit liver microsomal paraoxonase (MsPON) (15). We defined the substrate specificity of the purified rabbit serum PON3 over a number of esters and lactones and confirmed it with transiently expressed recombinant rabbit PON3. Of considerable interest is the close HDL association of serum PON3 and its enhanced ability compared with rabbit serum PON1 to protect LDL against oxidation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF220944.

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¹ The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; MsPON, rabbit liver microsomal paraoxonase; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; VLDL, very low density lipoproteins; HPGC, high performance gel filtration chromatography; apo, apolipoprotein; PAF-AH, platelet-activated factor acetyl hydrolase; RT-PCR, reverse transcriptase-polymerase chain reaction.
**TABLE I**

**Purification of rabbit serum PON3**

| Volume | Protein | Activity | Specific activity | Yield | Fold purification |
|--------|---------|----------|------------------|-------|------------------|
| Serum  | 155     | 7,000    | 2,294            | 0.33  | 100              |
| Blue-agarose | 72      | 108      | 835              | 7.8   | 37               |
| 1st DEAE | 40      | 10       | 326              | 32.6  | 14               |
| 2nd DEAE | 40      | 7.1      | 321              | 45.8  | 139              |
| Sephacryl 200 | 4       | 0.75     | 92              | 125.8 | 4               |
| Concanavalin A | 6      | 0.24     | 31              | 130.0 | 1.4               |

**EXPERIMENTAL PROCEDURES**

**Rabbit Serum PON3**

Rabbit blood was collected by ear vein bleeding and cardiac puncture of anesthetized (ketamine, 40 mg/kg intramuscular, and acepromazine, 1 mg/kg intramuscular) New Zealand White female rabbits. The blood was allowed to clot for 30 min at room temperature, and the serum was obtained after centrifugation for 15 min at 2,000 x g.

**Materials and Chemicals**—DEAE Bio-Gel A and SDS-PAGE low range protein molecular weight standards were obtained from Bio-Rad. Sephacryl 200 was purchased from Amersham Pharmacia Biotech. Cibacron Blue 3GA-agarose type 3000, concanavalin A-Sepharose 4B, Tergitol NP-10, and mevastatin (Compactin) were obtained from Sigma. Lovastatin (Mevacor®) and simvastatin (Zocor®) were purchased from Abbott. Tergitol NP-10, and mevastatin (Compactin) were obtained from Acros Chimica (Fair Lawn, NJ). All other lactones and esters used were purchased from Sigma-Aldrich. 

**Protein Assay and SDS-PAGE**—Protein was determined by its UV absorption at 280 nm or in samples containing Tergitol NP-10 by the BCA protein assay (Pierce), according to the manufacturer’s protocol. SDS-PAGE was performed as described previously (17), and the gels were stained with Coomassie Blue.

**Enzyme Purification**—The initial steps in the purification of rabbit serum PON3 essentially followed the procedure for purification of serum PON1 previously developed in this laboratory (6, 16). Rabbit serum was mixed batchwise with Cibacron Blue 3GA-agarose in 3 ml NaCl, 50 mM Tris/HCl buffer (pH 8.0), 1 mM CaCl₂, and 5 μl EDTA, poured into a column, and washed with the same buffer until the A₂₈₀ was below 0.3 A units and further with two column volumes of 25 mM Tris/HCl buffer (pH 8.0), 1 mM CaCl₂. The column was developed with 25 mM Tris/HCl buffer (pH 8.0), 1 mM CaCl₂, 20% glycerol, and 0.1% sodium deoxycholate; 10-mI fractions were collected at 1 ml/min. Fractions were monitored for both arylesterase activity (phenyl acetate hydrolysis) and lactonase activity (lovastatin hydrolysis) to localize PON1 and PON3, respectively. The two activities co-eluted after Blue-agarose chromatography of serum but were almost completely separated on a DEAE anion exchange column by elution with a linear 300-ml of 0–175 mM NaCl gradient in TCGT buffer (25 mM Tris/HCl (pH 8.0), 1 mM CaCl₂, 20% glycerol, and 0.1% sodium deoxycholate; 10-mI fractions were collected at 1 ml/min. Fractions with both arylesterase activity (phenyl acetate hydrolysis) and lactonase activity (lovastatin hydrolysis) to localize PON1 and PON3, respectively. 

**Results**

| Volume | Protein | Activity | Specific activity | Yield | Fold purification |
|--------|---------|----------|------------------|-------|------------------|
| 500    | 100     | 2.50     | 0.33             | 100   | 1                |
| 500    | 100     | 2.50     | 0.33             | 100   | 1                |
| 500    | 100     | 2.50     | 0.33             | 100   | 1                |
| 500    | 100     | 2.50     | 0.33             | 100   | 1                |
| 500    | 100     | 2.50     | 0.33             | 100   | 1                |

**Cloning of Rabbit PON3**—Total rabbit liver RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA) following the supplier’s protocol. RT-PCR was performed with the Titan One-tube RT-PCR system (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Primers for human PON3 (1), Px3–6 (GGCATAGAAGTCGGTCGTCGAAGACCC) and Px3–17 (GCTTCTGAAATGATTGATAATCCTCCAGTGGG) were designed. The RT-PCR products were separated on a 1% agarose gel. Bands of the expected size (as deduced by their similarity to the human PON3) were excised and submitted for sequencing in our DNA Sequencing Core Laboratory. Based on the obtained sequence, primers PX3–3 (CTTATCCTGTCGACCAAGTGGTG) and PX3–3 (ACACACGGCTCCGTCTTACG) were designed (these and all of the primers below were purchased from Life Technologies, Inc.) and used for amplification of the 5’- and 3’-ends of rabbit PON3 cDNA using a 5’-3’ rapid amplification of cDNA ends (RACE) kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Based on the sequence of the new primers of these fragments, PX3–3 (ATCGGAACTCATCGGCGGAGCCCTGCGG) and PX3–3 (AGGGCTTGACGGTGAACAGAG) were designed and used to amplify the full-length cDNA. The PCR product (~ 1200 base pairs) was cloned in the pcR® II vector with a TOPO®TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. PON3 clones from at least five animals were sequenced in both directions, and the consensus sequence was submitted to the GenBank® (accession number AF229944).

**Enzymatic Activities**—Esterase activities with substrates phenyl acetate, 5-phenyl thioacetate, α-naphthyl acetate, and paraoxon were measured as described elsewhere (6, 16).

Hydrolysis of aromatic lactones was monitored by the increase in UV absorbance at 270 nm (dihydrocoumarin), 274 nm (2-coumaranone), and 290 nm (homogentisic acid lactone). In a typical experiment a cuvette contained 1 mM substrate (from a 100 mM stock, dissolved in methanol) in 50 mM Tris/HCl (pH 8.0), 1 mM CaCl₂, and 5–20 μl of enzyme in a total volume of 1 ml. The molar difference extinction coefficients (difference between the absorption coefficients of the acid and the lactone) used to calculate the rate of hydrolysis were...
was loaded onto a Bio-Gel A-15m column (36 nm (22) using a quartz microtiter plate in a SPECTRAmax® 190 plate reader (Molecular Devices, Sunnyvale, CA). The lag time was estimated of PON3 or PON1 for up to 5 days at 37 °C. The kinetics of lipoprotein hydrolysis of the lactones in 0.02 M NaOH.

Products were calculated from the peak heights after complete alkaline catatin), and 4.4/6.6 min (simvastatin). Response factors for the acid substrate were as follows: 4.5/6.4 min (mevastatin), 4.4/6.6 min (lovastatin, and simvastatin, respectively. Under the above conditions of the following: A isocratically at a flow rate of 1.0 ml/min with a mobile phase consisting nitrile (100 m)

Human LDL was isolated by ultracentrifugation as described above and used within the next 3–4 days. LDL (0.1 mg protein/ml) oxidation was induced with 2–10 mM CuSO4 in phosphate-buffered saline (pH 7.4) in the absence or presence of PON3 and PON1 for up to 5 h at 37 °C. The kinetics of lipoprotein oxidation was followed by monitoring conjugated diene formation at 234 nm (22) using a quartz microtitr meter plate in a SPECTRAMAX 190 plate reader (Molecular Devices, Sunnyvale, CA). The lag time was estimated by drawing a perpendicular line to the x axis from the intersection of straight lines drawn through the absorption curves during the lag phase and the propagation phase, as illustrated on Fig. 4A. Lipoprotein oxidation was also determined by the lipid peroxides test, which analyzes lipid peroxides by their capacity to convert iodide to iodine, as measured photometrically at 365 nm (23).

RESULTS

Purification of a 40-kDa Protein with Lactonase Activity Figure 1. Purification of rabbit serum lactonase. A, Blue-agarose chromatography of rabbit serum. Arylesterase activity with phenyl acetate (solid circles), lactonase activity with lovastatin (open circles), and protein (+) were measured as described under “Experimental procedures.” B, the active fractions (107–114) from the previous step were applied onto a DEAE Bio-Gel anion exchange column equilibrated with TCGT buffer and eluted with 300 ml of 0–175 mM NaCl gradient. The symbols used are as described in A, but the protein concentration was measured by the BCA method. C, SDS-PAGE (12% acrylamide) of concanavalin A column fractions stained with Coomassie Blue. Pooled fractions from Sepharyl gel filtration column diluted 1:1 with 25 mM Tris/HCl buffer (pH 7.2), 1 mM CaCl2 (GFC pool) were passed through the concanavalin A (Conc. A) column and eluted with the same buffer. The lactonase activity was associated with the 40-kDa protein (indicated with an arrow).

The hydrolysis of other lactones was followed by a colorimetric assay with phenol red (5, 19). The reaction cuvette contained 1 mM substrate (from a 100 mM stock solution, dissolved in methanol) in 2 mM HEPES (pH 7.4), 1 mM CaCl2, 0.004% (106 µl) phenol red, 0.005% bovine albumin, and 5–20 µl of enzyme in a total volume of 1 ml. The rate of acid production was followed by monitoring the increase in absorbance at 422 nm. The rate of hydrolysis was derived from a calibration curve obtained using known amounts of HCl as described previously (19).

The enzymatic assays were performed at 25 °C using a dual beam Cary 3E UV/visible spectrophotometer (Varian, Australia). Reference cuvettes containing the appropriate buffer plus substrate were used in each assay to correct for any spontaneous hydrolysis.

The hydrolysis of the statin lactones (mevastatin, lovastatin, and simvastatin) was analyzed by HPLC using a Beckman System Gold HPLC with a model 126 programmable solvent module, a model 168 diode array detector set at 238 nm, a model 7125 flame manual injector valve with a 20-µl loop, and a Beckman ODS Ultrasphere column (C18, 250 × 4.6 mm, 5 µm). In a final volume of 1 ml, 10–200 µl of enzyme and 10 µl of substrate solution in methanol (0.5 mg/ml) were incubated at 25 °C in 25 mM Tris/HCl (pH 7.6), 1 mM CaCl2. Aliquots (100 µl) were removed at specified times and added to acetonitrile (100 µl), vortexed, and centrifuged for 1 min at maximum speed (Beckman Microfuge). The supernatants were poured into new tubes, capped, and stored on ice until HPLC analysis. Samples were eluted isocratically at a flow rate of 1.0 ml/min with a mobile phase consisting of the following: A = acetic acid/acetonitrile/water (2:249:249, v/v/v) and B = acetonitrile, in A/B ratios of 50/50, 45/55, and 40/60 for mevastatin, lovastatin, and simvastatin, respectively. Under the above conditions the retention times for the carboxylic acid formed and the lactone were prepared by sequential flotation ultracentrifugation to isolate very densities and by HPGC to confirm their identity and purity as described elsewhere (20). Lipoprotein Separation by Ultracentrifugation of Serum—Fractions were prepared by sequential flotation ultracentrifugation to isolate very low density lipoproteins (VLDL) (d < 1.019 g/ml), LDL (d = 1.019 to 1.063 g/ml), and HDL (d = 1.063 to 1.2 g/ml) (21). Lipoprotein fractions were dialyzed against phosphate-buffered saline (pH 7.4) containing 1 mM CaCl2 at 4 °C for 24 h, then kept at 4 °C in the dark. The isolated fractions were analyzed within the next 3–4 days for enzymatic activities and by HPGC to confirm their identity and purity as described (20).

Copper-induced LDL Oxidation in Vitro—Human LDL was isolated by ultracentrifugation as described above and used within the next 3–4 days. LDL (0.1 mg protein/ml) oxidation was induced with 2–10 µM CuSO4 in phosphate-buffered saline (pH 7.4) in the absence or presence of PON3 or PON1 for up to 5 h at 37 °C. The kinetics of lipoprotein oxidation was followed by monitoring conjugated diene formation at 234 nm (22) using a quartz microtitr meter plate in a SPECTRAMAX 190 plate reader (Molecular Devices, Sunnyvale, CA). The lag time was estimated by drawing a perpendicular line to the x axis from the intersection of straight lines drawn through the absorption curves during the lag phase and the propagation phase, as illustrated on Fig. 4A. Lipoprotein oxidation was also determined by the lipid peroxides test, which analyzes lipid peroxides by their capacity to convert iodide to iodine, as measured photometrically at 365 nm (23).

Rabbit Serum PON3

Purification of Rabbit Serum Lipoproteins by Gel Filtration—Serum was loaded onto a Bio-Gel A-15m column (36 × 1.8 cm) and eluted with 50 mM Tris/HCl (pH 8.0), 1 mM CaCl2. Fractions were collected and analyzed on a Hitachi 912 autoanalyzer (Roche Molecular Biochemicals) for phospholipids, triglycerides, and total and esterified cholesterol using commercially available kits from Wako Bioproducts (Richmond, VA). Representative fractions were combined, concentrated, and analyzed by high performance gel filtration chromatography (HPGC) to determine the lipoprotein cholesterol distribution as described elsewhere (20).

Lipoprotein Separation by Ultracentrifugation of Serum—Fractions prepared by sequential flotation ultracentrifugation to isolate very low density lipoproteins (VLDL) (d < 1.019 g/ml), LDL (d = 1.019 to 1.063 g/ml), and HDL (d = 1.063 to 1.2 g/ml) (21). Lipoprotein fractions were dialyzed against phosphate-buffered saline (pH 7.4) containing 1 mM CaCl2 at 4 °C for 24 h, then kept at 4 °C in the dark. The isolated fractions were analyzed within the next 3–4 days for enzymatic activities and by HPGC to confirm their identity and purity as described (20).

Coprophase-4 of the mammalian cell line—The purification of PON3 from rabbit serum is summarized in Table I. We used lovastatin hydrolysis (lactonase activity) and phenyl acetate hydrolysis (arylesterase activity) to follow purification of PON3 and PON1, respectively. During the Blue-agarose chromatography of serum, both activities co-eluted (Fig. 1A) but were resolved by DEAE anion exchange chromato-
teins with molecular masses of 50 and 63 kDa co-purified through all steps and became enriched during the purification (Fig. 1C, 2nd lane). The three proteins were transferred to polyvinylidene difluoride membrane, and each was submitted for N-terminal sequencing. The resolved peptide sequences are presented on Table II. The N terminal of the 40-kDa protein is identical with the deduced amino acids sequence of the rabbit PON3 cDNA we cloned (see below) and 96% identical with the amino acid sequence of MsPON (15). The N terminal of the 50-kDa protein is 73% identical with the deduced sequence (residues 22 to 36) of the human platelet-activated factor acetyl hydroxylase (PAF-AH) precursor (25). The N-terminal amino acid of the purified human PAF-AH is Ile-42, and the estimated size of the enzyme on SDS-PAGE is 44–45 kDa (25). Thus, the rabbit analogue of PAF-AH has 20 additional N-terminal amino acids, which could explain the observed difference in the molecular mass. The N terminal of the 63-kDa protein is 93% identical with both human and mouse vanin 1 (26). Both PAF-AH and vanin proteins are N-glycosylated (25, 26) and were retained by the concanavalin A column we used as a final step in the purification of PON3. The lactonase activity did not bind to the column, and the washout fractions showed a single band on SDS-PAGE with trace contamination of albumin and concanavalin A fragments (Fig. 1C, lanes 3–5).

The isolation of PON3 from its natural environment and the use of detergents, which was unavoidable because of the tight association of PON3 with other proteins, led to a decrease in its...
specific activity. Phospholipids, which were shown to stimulate the hydrolytic activity of purified PON1 (6), e.g. dilaurylphosphatidylcholine, were without effect on the PON3 lactonase activity (lovastatin hydrolysis), but adding apolipoprotein A-1 (apoA-1) in eighth-fold excess doubled it (data not shown).

Cloning of the Rabbit PON3 cDNA—We used the human PON3 sequence as a model for retrieving the rabbit PON3 cDNA as outlined under “Experimental Procedures.” We cloned a 1194-base pair cDNA with a 1065-base pair open reading frame (Fig. 2), encoding a protein of 39.5 kDa. This predicted mass is consistent with that determined for the purified lactonase by SDS-PAGE. In the mature protein, as previously shown for the rabbit and human PON1s (24), only the initial methionine is processed, and the remainder of the leader sequence is retained. The rabbit PON3 nucleotide sequence is 86 and 79% identical with those of human and mouse PON3, respectively, as well as 82 and 78% similar to their deduced amino acid sequences. Rabbit PON3 is 64/58% identical with the rabbit PON1 nucleotide/amino acids sequences (24). We cloned and sequenced rPON2 and found it 68/65% identical with rPON3 nucleotide/amino acids sequences.

Characterization of the Purified Rabbit Serum PON3—The purified PON3 hydrolyzed lovastatin with a specific activity of about 130 nmol/min/mg of protein. PON3 required Ca$^{2+}$ for this hydrolytic activity, which could be completely inhibited by EDTA. The lactonase activity was practically unaffected by millimolar concentrations of diisopropyl fluorophosphate or paraoxon (data not shown). The pH optimum for the lovastatin hydrolysis was estimated to be between pH 7.0 and 7.6 (data not shown). The rates of hydrolysis of simvastatin (73 nmol/min/mg) and mevastatin (95 nmol/min/mg of protein) were within the same range as lovastatin. In contrast, the hydrolytic activity of purified rabbit serum PON1 with lovastatin was less than 0.2 nmol/min/mg.

A comparison of purified rabbit serum PON3 and PON1 hydrolytic activities is presented in Table III. Phenyl acetate and thiophenyl acetate were poor substrates for PON3, whereas the rate of hydrolysis with dihydrocoumarin, 2-coumarone, and some 5- and 6-member ring lactones with aliphatic substituents was up to 2 orders of magnitude higher. In contrast to PON1, PON3 had no activity with unsubstituted lactones or with 4- and 7-member ring lactones. PON3 required at least one methylene group in the 5- or 6-position (for the γ- or δ-lactones, respectively), and its lactonase activity increased with the number of carbon atoms in the aliphatic chain (up to 5 for the γ-lactones and up to 7 for the δ-lactones). Compared with PON3, rabbit PON1 exhibited more potent lactonase activity and broader substrate specificity, but no single trait in the substrate structure-enzyme activity relationship could be identified. Thus, the PON1/PON3 lactonase activity varied from 0.9–4-fold up to 52–97-fold (Table III). Paraoxon was not hydrolyzed by PON3.

Transient Expression of Recombinant Rabbit Pon3—293T/17 cells transfected with rabbit PON3 cDNA in pcDNA3.1(+) expressed and secreted PON3, as evaluated by the increase in the lactonase activity (lovastatin hydrolysis) of the expression medium. The highest lactonase activity (2.8 ± 0.2 nmol/ml of medium, average from four 10-cm culture dishes) was obtained 4–5 days after transfection. The expression medium from 293T/17 cells transfected only with the pcDNA3.1(+) vector had no lactonase activity. The activity of the lysate from PON3-transfected cells harvested 5 days after transfection accounted for only 25% of the total lactonase activity, suggesting efficient secretion of the enzyme. Expression medium from PON3-transfected cells was chromatographed on a small Blue-agarose column. The recombinant PON3 co-eluted with apoA-1 and albumin (from the supplemented medium) and had the same apparent molecular mass on SDS-PAGE as PON3 purified from rabbit serum (data not shown). The hydrolytic activity of the Blue-agarose fraction, containing the recombinant PON3, was 6.5 nmol/min/ml (lovastatin), 4.6 μmol/min/ml (dihydrocoumarin), and 0.043 μmol/min/ml (phenyl acetate). Paraoxon was not hydrolyzed by this fraction.

HDL Association of PON3—The co-purification of PON3 with apoAI and PON1 through the Blue-agarose chromatography step (Fig. 1A) suggested that both PONs are associated with the serum HDL. In another experiment, rabbit serum lipoproteins were separated by gel filtration chromatography. VLDL eluted in fractions 10–14, LDL in 18–28, and HDL in 29–52, respectively (Fig. 3A). The purity and the identity of the lipoprotein fractions were evaluated by HPGC of the lipoprotein cholesterol (Fig. 3B). The arylesterase activity was recovered in fractions 30–46, and the lactonase activity (lovastatin) was recovered in fractions 29–49 (Fig. 3C). Thus, both activities co-eluted with the HDL fractions.

Rabbit serum lipoproteins were also separated by sequential flotation ultracentrifugation (Table IV). Virtually all of the recovered lactonase activity (PON3) as well as all arylesterase and paraoxonase activities (PON1) were in the HDL fraction. The higher recovery of PON3 (22%) compared with PON1 (13%) during ultracentrifugation could be due to tighter binding to HDL and/or greater stability of PON3.

Protection of LDL against Lipid Peroxidation—The protective role of purified human PON1 against in vitro copper-induced oxidation of HDL and LDL has been well established (7–10, 12, 28). Initial experiments in our laboratory showed

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**Table III**

| Substrate PON3 | PON1 | PON1/PON3 |
|----------------|------|-----------|
| **Esters**     |      |           |
| Phenyl acetate | 1.0  | 769       |
| Thiophenyl acetate | 0.48 | 259 | 540 |
| β-Naphthaldehyde | 4.6  | 139 | 30 |
| Paraoxon (at pH 8.0) | 0    | 4.76 |
| **Aromatic lactones** |      |           |
| Dihydrocoumarin | 220  | 192 | 0.9 |
| 2-Coumarone     | 32.4 | 137 | 4.0 |
| Homogentisic acid lactone | 2.8  | 270 | 97 |
| **γ-Lactones**  |      |           |
| γ-Valeralactone | 3.0  | 78.4 | 26 |
| γ-Hexalactone  | 4.6  | 96  | 21 |
| γ-Heptalactone | 8.6  | 107 | 12 |
| γ-Octalactone | 29   | 104.5 | 3.6 |
| γ-Nonalactone | 60   | 79.6 | 1.3 |
| γ-Decanolactone | 50  | 92  | 1.8 |
| γ-Undecanolactone | 44 | 87.4 | 2.0 |
| **β-Lactones**  |      |           |
| β-Methyl-γ-octalactone | 3.0 | 34.8 | 12 |
| β-Methylene-γ-butyrolactone | 12.0 | 622 | 52 |
| α-Angelica lactone | 3.4  | 175 | 52 |
| **δ-Lactones**  |      |           |
| δ-Hexalactone | 3.6  | 207 | 58 |
| δ-Nonalactone | 44   | 142 | 3.4 |
| δ-Decanolactone | 73  | 135 | 1.8 |
| δ-Undecanolactone | 90 | 187 | 2.1 |
| δ-Dodecanolactone | 98  | 179 | 1.8 |

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2. C. Watson, unpublished data.
that both purified rabbit PON1 and PON3 delayed LDL oxidation in a concentration-dependent manner; however, PON3 had a much more pronounced effect than PON1. The results of experiments in which the effects of 0.1–2.5 μg of PON3 (10–250 nmol/liter) were compared with those of 10 and 20 μg of PON1 (1 and 2 μmol/liter) are presented in Fig. 4. PON3 (0.5 μg; 50 nmol/liter) added to the incubation mixture doubled the lag time for conjugated diene formation and reduced significantly the amount of lipid peroxides generated at all time points of observation. The effect of 0.1 μg of PON3 on conjugated diene formation and lipid peroxide accumulation was between those obtained with 10 and 20 μg of PON1. Thus, rabbit PON3 is more than 100 times more potent per μg of protein than rabbit PON1 in protecting LDL against copper-induced oxidation.

DISCUSSION

Purified rabbit serum PON3 has a molecular mass of 40 kDa on SDS-PAGE, in agreement with the predicted value from the deduced amino acid sequence of the PON3 cDNA. Like PON1, rabbit serum PON3 retains most of its hydrophobic leader sequence, and only the initial methionine is cleaved. This portion of PON1 is responsible for its tight binding to HDL phospholipids (29), and it is reasonable to assume that this also accounts for the association of rabbit serum PON3 with HDL. In this study we separated rabbit serum lipoproteins by gel chromatography and by ultracentrifugation. Virtually all of the PON3 hydrolytic activity was recovered with the HDL fractions (Fig. 3 and Table IV). Immunopurified PON1 is associated with two major proteins: apoA-I and apoJ (30, 31), but PON3 seems to be more widely distributed than PON1 in the gel filtration HDL fractions (Fig. 3). Further experiments will be needed to study the association of PON3 with particular HDL subclass and/or particular apolipoproteins. Rabbit liver MsPON appears to lack the tripeptide Ala-Lys-Leu at its N terminus (15). At present, it is not clear whether this difference is an artifact arising during purification or the result of different processing. Thirteen additional differences were found between the reported MsPON amino acid sequence (15) and that deduced from rabbit PON3 cDNA (Fig. 3). Recently, we observed that commercially available New Zealand White rabbits show at least eight-nucleotide differences in the coding region of the PON1 gene, resulting in five amino acid changes as well as in a polypeptide four amino acids shorter at the C terminus. A similar number of changes in the neighboring PON3 gene and/or an additional PON3 gene in rabbits may account for the differences between the PON3 sequence we found and that reported for MsPON (15).

Rabbit serum PON1 and PON3 share considerable amino acid sequence identity (58%) but show distinct substrate specificities. PON1 hydrolyzes paraoxon and aromatic esters (2, 4) as well as many aromatic and aliphatic lactones (Refs. 4 and 5 and this study). Although the active site of the PON1 has not been established, some residues essential for the hydrolytic

C. Watson, personal communication.
activity have been identified by both chemical modification and site-directed mutagenesis (32–34). The chemical modification of tryptophan, histidine, aspartic acid/glutamic acid, or cysteine residues reduced or abolished enzymatic activity. Sixteen of these residues in human PON1 were identified as essential for the hydrolytic activity, and all of them except His-243 and Trp-281 are conserved in rabbit PON3. The two PON1 mutants, W281L and H243K, corresponding to the PON3 sequence, have markedly reduced arylesterase and organophosphatase activity (32, 33). This observation may at least in part explain the limited arylesterase and paraoxonase activity of rabbit serum PON3 we observed. Both PON3 and PON1 have lactonase activity, but PON1 hydrolyzes a much broader spectrum of lactones at higher rates than PON3. Six-member ring lactones were better substrates than their five-member homologs for both PON3 and PON1. Interestingly, rabbit serum PON3 hydrolyzes the statin lactones at a much higher rate than PON1. During the purification of rabbit serum PON3 and PON1, we found that the PON1 is about 200 times more abundant than PON3, but nevertheless, the overall contribution of PON1 to rabbit serum lactonase activity (lovastatin hydrolysis) is less than 1%, as calculated from the specific activity of the purified enzyme. It has been shown that lovastatin and simvastatin are rapidly hydrolyzed by mouse and rat plasma and very slowly by human and dog plasma (35, 36), whereas simvastatin is hydrolyzed by rabbit plasma at rates between those of rodent and the human plasma (36). However, we found that about 95% of the mouse and rat serum lactonase activity (with lovastatin) does not bind to Blue-agarose and is completely inhibited by diisopropylphosphofluoridate or paraoxon but not by EDTA.4 Thus, it is more likely that in rodents carboxylesterase(s) account for this activity. It has been shown that lactonase activity in rodent and human serum, it is even less abundant than in rabbit serum and/or has lower lactonase activity with the statin prodrugs than rabbit serum PON3.

A physiological role for PON1 and the other PON enzymes is still unknown. However, epidemiological and biochemical evidence is accumulating that suggests an important role for PON1 against atherosclerosis (7–9). PON1 protects both LDL and HDL from oxidation (28, 38, 39), probably related to its ability to hydrolyze specific oxidized phospholipids (7, 10) and cholesteryl linoleate hydroperoxides (28, 39, 40). The ability of PON1 to protect LDL against oxidation is paralleled by inactivation of the enzyme, which results from an interaction of oxidized phospholipids and cholesteryl esters with the PON-free sulfhydryl group (39). Although human and rabbit PON1 have about the same ability to protect LDL against in vitro copper-induced oxidation,4 we unexpectedly found that rPON3 is about 100-fold more potent than rPON1 (Fig. 4). We propose

### Table IV

| Lipoprotein Fraction | Enzyme Activity | Recovery (%) |
|----------------------|-----------------|-------------|
| Serum                | Arylesterase    | 100         |
| VLDL                 | Specific activity | 14.2        |
| LDL                  | Total activity | 17,600       |
| HDL                  | Specific activity | 14.2        |
|                      | Recovery (%)    | 100         |
|                      | Arylesterase    | 0.02        |
|                      | Specific activity | 0.02        |
|                      | Recovery (%)    | 100         |

**FIG. 4.** Effect of purified PON3 and PON1 on in vitro copper-induced LDL oxidation. LDL (100 µg/ml) were incubated at 37 °C in phosphate-buffered saline (pH 7.4) in the presence or absence of purified PON3 or PON1 as indicated in the legends. Lipid peroxidation was initiated with 5 µM CuSO4. A, accumulation of conjugated dienes measured as an increase in the absorbance of the samples at 234 nm after the addition of Cu2+ and calculated lag times (inset panel). B, effect on lipid peroxide formation. Lipid peroxides were measured as described in El-Saadani et al. (23). The data are shown as the mean ± S.D. for triplicates from a representative out of three experiments with similar results. There were no detectable levels of lipid peroxides in the LDL before oxidation. * and ** denote significant difference from the control at p < 0.05 and p < 0.01, respectively.

4 D. I. Draganov, P. L. Stetson, C. E. Watson, S. S. Billecke, and B. N. La Du, unpublished results.

4 H. P. Kieft, J. T. van der Grinten, and J. P. M. van der Veen, unpublished results.
that rPON3 acts by a mechanism similar to that of PON1. Further experiments are needed to find the reason(s) for better protection by rPON3 against LDL oxidation, which might be due to different substrate specificity, kinetic properties and/or stability of the two enzymes. We estimate that rPON3 is present in serum at concentrations of about 20–50 nmol/liter, whereas the concentration of rPON1 in serum is about 5 µmol/liter. If the in vitro data we report here reflects the in vivo activity of PON3 in rabbits, its contribution against LDL oxidation might be as great as that of rPON1.

Northern blot analyses had previously detected PON1 mRNA only in rabbit and human liver (24). Using RT-PCR, PON1 mRNA was detected in adult mouse liver, kidney, heart, brain, small intestine, and lung but not in pancreas nor in white blood cells (1), whereas PON2 and PON3 mRNAs were detected in all of the tissues studied (1). Based on the expressed sequence tag clones representation in GenBankTM, it appears that PONs differ in their tissue expression and distribution; human and mice PON1-expressed sequence tags originate from liver, lung, and brain, PON2-expressed sequence tags are expressed ubiquitously, and PON3 is represented somewhere in between. To date, there are no data about the secretion of PON2 and PON3 in other species than rabbit (this report).

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