

## Directed Evolution of Serum Paraoxonase PON3 by Family Shuffling and Ancestor/Consensus Mutagenesis, and Its Biochemical Characterization<sup>†</sup>

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**ABSTRACT:** Serum paraoxonases (PONs) are calcium-dependent lactonases with anti-atherogenic and detoxification functions. Here we describe the directed evolution and characterization of recombinant variants of serum paraoxonase PON3 that express in an active and soluble manner in *Escherichia coli*. These variants were obtained by combining family shuffling and phylogeny-based mutagenesis: the limited diversity of accessible, cloned PON3 genes was complemented by spiking the shuffling reaction with ancestor/consensus mutations, mutations to residues that comprise the consensus or appear in the predicted ancestors of the PON family. We screened the resulting libraries for PON3's lactonase activity while ensuring that the selected variants retained the substrate specificity of wild-type mammalian PON3s. The availability of highly stable, recombinant PON3 that is free of all other serum components enabled us to explore unknown biochemical features of PON3, including its binding to HDL particles, the effect of HDL on PON3's stability and enzymatic activity, and ex vivo tests of its anti-atherogenic properties. Overall, it appears that PON3 possesses properties very similar to those of PON1: the enzyme's lactonase activity is selectively stimulated by binding to apoAI-HDL, with a concomitant increase in its stability. PON3 also exhibits potentially anti-atherogenic functions, although at levels lower than those of PON1.

Serum paraoxonases (PONs)<sup>1</sup> are calcium-dependent mammalian enzymes that have been recently characterized as lipophilic lactonases (1, 2). PONs exhibit a range of biologically important activities, such as organophosphate hydrolysis and protection against atherosclerosis, although their physiological substrate(s) remains unknown. PON1 is the only well-characterized member of the family, the other members being PON2 and PON3. While PON1 is present mostly in the serum bound to HDL, PON2 present in all the tissues but not on HDL, while PON3 resides both on HDL and in various tissues (1, 3).

PON3 has been studied much less than PON1. Its structure is unknown, nor was it engineered, primarily due to lack of ample sources of stable, bacterially expressed PON3 variants similar to those that permitted extensive exploration of PON1 (1, 4). It

shares 65% amino acid identity with PON1 and exhibits substantial lactonase activity. However, its promiscuous esterase and phosphotriesterase activities are much weaker than those of PON1 (1). Although it is known that PON3 has an anti-atherogenic potential (5–8), little is known about its biochemical properties.

Before it became apparent that PONs are lactonases (1, 2), both PON1 and PON3 were subjected to directed evolution for *Escherichia coli* expression (4), using family shuffling and screens for the aryl-esterase and paraoxonase activities (that are now known to be promiscuous). In both cases, variants with a higher level of bacterial expression were selected. While in PON1 their enzymatic parameters fortuitously remained as they are for the wild type, serum-purified PON1 (including for lipophilic lactones that were not screened for at the time), improvement in bacterial expression of PON3 was accompanied by significant changes in substrate specificity, including >20-fold increases in aryl-esterase activity and >200-fold increases in phosphotriesterase activity. These recombinant PON3 variants were, therefore, unsuitable for mechanistic and biochemical studies aimed at improving our understanding of PON3's native functions.

We therefore aimed to perform directed evolution of PON3 while screening for the lactonase activity, assuming that such a procedure would yield the desired bacterial expression without changing the enzymatic properties. The lipophilic lactones

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<sup>1</sup>Abbreviations: PON, serum paraoxonase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TBBL, 5-thiobutylbutyrolactone; TEBL, 5-thioethylbutyrolactone; THBL, 5-thiohexylbutyrolactone; WT, wild-type.

hydrolyzed by PONs are not suitable for high-throughput screening (1, 9, 10), and we therefore applied chromogenic lactone substrates, 5-thioalkyl butyrolactones (TXBLs) (11). Throughout the process, variants with increased lactonase activity [assayed primarily with 5-thiobutyl- $\gamma$ -butyrolactone (TBBL)] were also assayed for their esterase and phosphotriesterase activities, and their expression levels were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In this way, only the variants with enhanced expression and no apparent changes in enzymatic specificity were selected.

For the creation of genetic diversity, we used family shuffling of three orthologous PON3 genes. Family shuffling has been widely used in directed evolution to increase stability and the level of bacterial expression, and to alter substrate specificity (12, 13). However, family shuffling has certain limitations, the most obvious being that in most cases, only a limited range of family members is available. We cloned human, mouse, and rabbit PON3 but failed to clone rat PON3. In addition, as seen for PON3, family shuffling alone may not yield a sufficiently stable enzyme (14). However, the outcome of family shuffling can, in effect, be equivalent to consensus mutagenesis. Indeed, homology-driven strategies are routinely used for engineering higher stabilities (15). Mutagenesis of residues that differ from the consensus to the most frequently occurring residue within the family often leads to higher stabilities (16–19). Ancestors predicted by phylogenetic analysis were also shown to be more thermostable than contemporary family members (20–22). Indeed, our analysis of the PON1 variants that were evolved for bacterial expression indicated that family shuffling led to the fixation of consensus/ancestor mutations. Thus, we explored the possibility of complementing family shuffling with consensus/ancestor mutations that are incorporated into the shuffled genes in a combinatorial manner. This combined strategy enabled the evolution of stable PON3 variants that express in *E. coli* at high levels (> 30 mg/L of culture). The availability of highly stable, recombinant PON3 that is free of all other serum components enabled us both to explore several biochemical features of PON3 and to examine the anti-atherogenic potential of this relatively unexplored member of the PON family.

## EXPERIMENTAL PROCEDURES

**Library Construction.** *Round 1.* The wild-type PON3 genes of human, rabbit, and mouse, cloned in the pET32b(+) vector (4), were individually amplified, mixed in equal amounts, and digested with DNase I (Sigma). The resulting 50–200 bp fragments were assembled by polymerase chain reaction (PCR) as described previously (12). The resulting libraries were cloned into the pET32b(+) vector (Novagen) using *Nco*I and *Not*I restriction sites, expressing the PON3 genes at the C-terminus of thioredoxin for the increased solubility (4). The ligated DNA was transformed into *E. coli* DH5 $\alpha$  cells, yielding  $\sim 10^5$  transformants, and the plasmid DNA was extracted to obtain the library of this round and subsequent rounds. Sequencing indicated 8–10 crossovers per gene, and fewer than one random mutation per gene was introduced by the PCR procedures.

*Round 2.* The best variants from round 1 (19 in total) were mixed in equal proportions, shuffled, and recloned as described above.

*Round 3.* The best variants of round 2 (nine in total) were recloned into the pET32 vector with a C-terminal His tag and without the thioredoxin at the N-terminus (pET32-trx). The variants in pET32-trx were PCR amplified, mixed in equimolar

amounts, shuffled in the presence of 30% wild-type rabbit PON3 for backcrossing, and recloned into pET32-trx.

*Round 4.* The best variants of round 3 (11 in total) were mixed in equal proportions, shuffled in the presence of 25% wild-type rabbit PON3, and recloned into pET32-trx.

*Round 5.* The three best variants from round 3, and the four best variants from round 4, were mixed in equal proportions, amplified, and digested with DNase I. The 50–200 bp fragments were PCR assembled in the presence of an equimolar mixture of 12 oligonucleotides encoding the ancestor/consensus mutations [10 pmol in total (Table 1 of the Supporting Information)] as described previously (23). The assembled DNA was amplified and cloned into pET32-trx.

After five rounds, the random PCR mutations that did not originate from the starting genes, nor from the spiking oligonucleotides, had accumulated to a level of  $2 \pm 1$  per gene.

**Screening.** Plasmid DNA was transformed into *E. coli* BL21 DE3 cells (round 1) or *E. coli* origami B DE3 cells (rounds 2–5) and plated on LB agar plates containing 100  $\mu$ g/mL ampicillin. Individual colonies were inoculated into 2YT medium supplemented with 100  $\mu$ g/mL ampicillin and 1 mM CaCl<sub>2</sub> in 96-deep well plates (300  $\mu$ L/well) and grown overnight at 30 °C. Overnight cultures (20  $\mu$ L) were inoculated into 2YT medium supplemented with 100  $\mu$ g/mL ampicillin and 1 mM CaCl<sub>2</sub> (500  $\mu$ L) in 96-deep well plates and grown at 30 °C to an OD of  $\sim 0.6$ . Overexpression was induced by adding 1 mM IPTG; the cultures were grown for 5 h at 25 °C and then for  $\sim 20$  h at 20 °C and centrifuged, and the pellets were stored overnight at  $-20$  °C. The cells were lysed with lysis buffer [50 mM Tris (pH 8), 1 mM CaCl<sub>2</sub>, 0.2% Triton 100, and 0.1 mg/mL lysozyme, at 250  $\mu$ L/well], and the lysates were clarified by centrifugation. The lysates were screened for lactonase activity with 5-thiobutylbutyrolactone (TBBL, 0.2 mM) (11) by monitoring the released thiol moiety with DTNB (Ellman's reagent) at 412 nm in a Power HT microtiter scanning spectrophotometer. The variants exhibiting the highest lactonase activity were then tested with ester (2-naphthyl acetate, 0.2 mM, detection at 320 nm) and phosphotriester (paraoxon, 0.5 mM, detection at 405 nm) substrates. Variants displaying enhanced expression (as judged by SDS–PAGE) and no change in substrate specificity were taken for the next round. To ensure monoclonality, and to verify the activity of the selected variants, the enzymatic activities were reassayed after growing two subclones from each original colony. Plasmid DNA of the verified subclones was extracted and used for sequencing and then taken as the templates for the subsequent round of mutagenesis and screening.

**Protein Expression and Purification.** The variants were retransformed into *E. coli* origami B DE3 cells. Five milliliters of 2YT medium with 100  $\mu$ g/mL ampicillin and 1 mM CaCl<sub>2</sub> were inoculated with a single colony and grown at 30 °C for  $\sim 15$  h. 2YT medium with 100  $\mu$ g/mL ampicillin and 1 mM CaCl<sub>2</sub> (500 mL) was inoculated with 5 mL of overnight culture and grown at 30 °C to an OD<sub>600</sub> of  $\sim 0.6$ . Overexpression was induced by adding IPTG (1 mM), and the cultures were grown for 5 h at 25 °C and then for  $\sim 20$  h at 20 °C. The cells were isolated by centrifugation and the pellets stored overnight at  $-20$  °C. The cells were resuspended in lysis buffer [50 mM Tris (pH 8.0), 1 mM CaCl<sub>2</sub>, 50 mM NaCl, 0.1 mM DTT, and 5  $\mu$ M EDTA] and lysed by sonication. The lysate was supplemented with 0.1% zwittergent (Calbiochem) and gently stirred for 3 h at 4 °C. The soluble fraction was treated with ammonium sulfate [55% saturation (w/v) at 0 °C]. The precipitate was resuspended and dialyzed

once against lysis buffer supplemented with 0.1% zwittergent and then against activity buffer [50 mM Tris (pH 8.0), 50 mM NaCl, and 1 mM CaCl<sub>2</sub>] containing 0.1% zwittergent.

After dialysis, the protein was bound to Ni-Nta resin (Ni-Nta His-Bind Resin, Novagen), washed first with activity buffer with 0.1% zwittergent and then with 10 and 20 mM imidazole in activity buffer with 0.1% zwittergent, and eluted with 150 mM imidazole in activity buffer with 0.1% zwittergent.

The Ni-Nta active pool was dialyzed against buffer A [20 mM Tris (pH 8.0), 1 mM CaCl<sub>2</sub>, and 0.1% zwittergent] and further purified by ion-exchange chromatography. The protein was applied on a HiTrap Q HP 5 mL column (GE Healthcare) and eluted with a linear gradient from 26 to 33% buffer B (buffer A with 1 M NaCl). Fractions with the highest lactonase activity were analyzed for purity on a 12% SDS-PAGE gel, pooled, concentrated to 4 mL on Vivaspin device with a 10 kDa molecular mass cutoff, and finally purified by size-exclusion chromatography (HiLoad Superdex 200 pg 26/60 column, GE Healthcare) using 20 mM Tris (pH 8.0), 1 mM CaCl<sub>2</sub>, 300 mM NaCl, and 0.1% zwittergent for elution. Fractions with the highest lactonase activity were analyzed for purity on a 12% SDS-PAGE gel, pooled, and dialyzed against storage buffer [50 mM Tris (pH 8.0), 1 mM CaCl<sub>2</sub>, 50 mM NaCl, and 0.1% zwittergent]. Sodium azide (0.02%) was added for storage at 4 °C, and protein concentrations were determined using the BCA protein assay kit (Pierce).

For the determination of activity units, only purification on the Ni-Nta column was performed. For kinetic studies, HDL experiments, and anti-atherogenic experiments, the full purification protocol was applied.

**Detergents.** After several detergents were examined (Table 2 of the Supporting Information), zwittergent was selected for all biochemical studies and for future screening of crystallization conditions. The activity of PON3 in a buffer supplemented with 0.1% zwittergent was high and stable over time, and the detergent could be easily removed by dialysis. The micelles of PON3 obtained with zwittergent were relatively small, which indicated a lack of aggregation.

**Rabbit PON3.** Wild-type rabbit PON3 was cloned, expressed, and partially purified as described previously (24). The expression levels were very low (< 2 mg/L of culture), unstable (in many cases almost no active enzyme was obtained), and entirely dependent on the presence of the thioredoxin fusion. Purification resulted in only ~30% pure enzyme as judged by SDS-PAGE. Nonetheless, a kinetic analysis was performed since no contaminating lactonase, esterase, or paraoxonase activities were observed. The ratios between the various activities of rabbit PON3 were therefore taken as a reference, and not their absolute values.

**Kinetic Measurements.** The kinetic measurements were performed in activity buffer [50 mM Tris (pH 8), 1 mM CaCl<sub>2</sub>, and 50 mM NaCl]. A range of enzyme concentrations were used, depending on the reactivity of the substrate, and the protein. The activity of PON3 variants was examined with the commercially available substrates paraoxon, 2-naphthyl acetate, phenyl acetate, *p*-nitrophenyl acetate, butyrate, and octanoate, dihydrocoumarin, 2-coumaranone, and aliphatic  $\gamma$ - and  $\delta$ -lactones with side chains of various lengths. TEBL, TBBL, and THBL were obtained and assayed as described previously (11), and hydrolysis of the aliphatic lactones was monitored as previously described (2). Substrate concentrations had to be varied according to the solubility, reactivity, and extinction coefficient of each substrate. The hydrolyses of lovastatin and estradiol 3,17-diacetate were monitored by high-performance liquid chromatography

(HPLC) as described previously (9, 25) (C8 column, isocratic 55% acetonitrile/H<sub>2</sub>O, with 0.2% acetic acid for lovastatin, and isocratic 70% acetonitrile/H<sub>2</sub>O with 0.2% acetic acid for estradiol 3,17-diacetate). For determination of the kinetic parameters, substrate concentrations ranged from  $0.3 \times K_M$  to  $2-3 \times K_M$ . The percentage of cosolvent (MeOH for phenyl acetate and paraoxon, DMSO for aliphatic lactones, and acetonitrile for TXBLs) was set to 1 or 1.6% in all the reactions with a given substrate. The reported values of the specific activity units and of the kinetic parameters represent the averages of at least two independent measurements.

**Data Analysis.** Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation [ $v_0 = k_{cat}[E]_0[S]_0 / ([S]_0 + K_M)$ ] using Kaleidagraph version 5.0. In cases where solubility limited substrate concentrations, data were fitted to the linear regime of the Michaelis-Menten model ( $v_0 = [S]_0[E]_0 k_{cat} / K_M$ ) and  $k_{cat} / K_M$  was deduced.

**Stability Studies.** Samples of PON3 round 5 1/10A and 3/11B (~4  $\mu$ M) were delipidated using an excess of Bio-Beads SM-2 (Bio-Rad) (typically, a 4 h incubation of a 100  $\mu$ L protein sample with 20 mg of rinsed beads). The delipidated samples were diluted 10-fold in 10  $\mu$ M reconstituted HDL (rHDL) (26) in activity buffer [50 mM Tris (pH 8.0), 1 mM CaCl<sub>2</sub>, and 50 mM NaCl] and incubated for 1 h at 37 °C. The same samples were diluted in activity buffer as a control. We initiated inactivation by adding an equal volume of inactivation buffer [2 mM EDTA in 50 mM Tris (pH 8.0)] and incubating the samples at 37 °C. Unlike the protocol used with PON1 (26), no reducing agent ( $\beta$ -mercaptoethanol or DTT) was used, since monitoring of TBBL hydrolysis involves detection of free thiol groups. Aliquots were taken at suitable time points and diluted in activity buffer, and the residual activity was determined with TBBL (0.25 mM). Inactivation rates were fitted to either single- or double-exponential fits (26):  $R = A_1 \exp(-k_1^{\text{inact}} t) + A_2 \exp(-k_2^{\text{inact}} t)$ , where  $R$  is the residual activity (expressed as the percentage of the initial activity of the delipidated enzyme),  $A$  is the amplitude of each of the single-exponential phases, and  $k$  is the rate constant of inactivation for that phase. Subscripts 1 and 2 designate the first (fast) and second (slow) phases of inactivation, respectively.

**Stimulation of PON3 Activity by rHDL.** Delipidated PON3 variants (~1  $\mu$ M) were incubated with a range of rHDL (0–7.5  $\mu$ M) or zwittergent (0–0.1%) concentrations, in TBS [20 mM Tris (pH 7.5) and 150 mM NaCl] containing 1 mM CaCl<sub>2</sub> for 3 h at 30 °C. Enzymatic activity was determined with TBBL (0.25 mM), with  $\gamma$ -undecanoic lactone 0.5 mM), and with *p*-nitrophenyl acetate (0.5 mM).

**Anti-Atherogenic Properties of PON3.** (i) **Cells.** J774A.1 murine macrophage cells were purchased from American type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal calf serum.

(ii) **Macrophage-Mediated LDL Oxidation.** LDL (purified from human sera; 100  $\mu$ g of protein/mL) was incubated with J774A.1 macrophages for 3 h in RPMI medium (without phenol red) in the presence of 5  $\mu$ M CuSO<sub>4</sub>. rePON3 1/10A, rePON3 3/11B, and rePON1 G3C9 (4) preassociated with rHDL (50  $\mu$ g of HDL protein) were added to the incubation medium of the LDL with the cells. LDL samples were also incubated in a cell-free system (27). The extent of LDL oxidation by the cells was measured by the thiobarbituric acid reactive substances (TBARS) assay (28). The values obtained in the cell-free system were subtracted from the values obtained with the cells.

(iii) *rHDL-Mediated Cholesterol Efflux from Macrophages*. J774A.1 macrophages were preincubated for 1 h with [ $^3\text{H}$ ]cholesterol, followed by cell wash and further incubation for 3 h at 37 °C, without rHDL or with rePON3 or rePON1 preassociated with rHDL (50  $\mu\text{g}$  of HDL protein). The cell medium was collected; cells were washed three times with PBS, and 0.1 M NaOH was added. Cell and medium  $^3\text{H}$  labels were quantified, and the percentage of cholesterol efflux was determined as medium  $^3\text{H} \times 100 / (\text{medium } ^3\text{H} + \text{cell } ^3\text{H})$ . The cholesterol efflux rate for the cells incubated without rHDL was 3.5%, and this value was subtracted from the values observed in the presence of rHDL (29).

## RESULTS

*Directed Evolution for an Increased Level of Bacterial Expression*. Three wild-type PON3 genes (rabbit, human, and mouse, 81–87% amino acid identity) were cloned in fusion with thioredoxin (for increased solubility) and transformed into *E. coli*. Only rabbit PON3 gave soluble, functional enzyme, albeit at very low and highly fluctuating levels (< 2 mg of active enzyme/L of culture). The genes were shuffled, and the resulting libraries were screened for lactonase activity with 5-thiobutylbutyrolactone (TBBL). The variants exhibiting the highest lactonase activity were tested with ester and phosphotriester substrates (2-naphthyl acetate and paraoxon). The crude lysates were also analyzed by SDS–PAGE for the levels of aggregated versus soluble PON3, to ensure that the increase in lactonase activity was correlated with an increase in the level of soluble expression of PON3 (Table 1 and Figure 1). The ratios among the lactonase, esterase, and phosphotriesterase activities were compared to those of wild-type rabbit PON3. Variants with enhanced expression, but with no changes in enzymatic specificity, were selected. Indeed, the substrate specificity of PON3 was easily changed, and shifts of up to 10-fold in relative activities (esterase/lactonase and/or phosphotriesterase/lactonase) were often observed. Typically, in each round, ~1000 clones were screened for lactonase activity, 20–40 variants with increased lactonase activity were selected, and only a minority of those (< 10) exhibited the original substrate specificity. The specificities of evolved PON3 variants isolated throughout the process are summarized in Table 1.

Following the second round of shuffling, several variants with a significant increase in their level of expression [ $> 30$  mg/L of culture (Figure 1)] and with no significant changes in specific activities were obtained. Albeit, these PON3 variants were screened, expressed, and purified with thioredoxin fused to their N-termini. There is, however, evidence that PON3 can be associated with HDL, and its binding to HDL particles is likely to be mediated by the N-terminal helix as is the case for PON1 (26). Thus, we aimed to evolve PON3 variants that can be expressed with an uninterrupted N-terminus (i.e., without the thioredoxin tag). The best round 2 variants were, therefore, recloned into a pET32 plasmid with a His tag attached at the C-terminus and with no tag at the N-terminus, which resulted in a significant decrease in the level of soluble expression (Figure 1). Two more rounds of shuffling and screening were performed, which produced several improved variants. However, although round 4 PON3 variants without the thioredoxin tag could be purified, they were obtained in relatively low yields and exhibited a strong tendency to aggregate. The round 4 variants also showed a high degree of convergence (94–98% identity between round

Table 1: Substrate Specificities and Expression Levels of Evolved PON3 Variants

	expression (mg of protein/L of bacterial culture)	lysate activity with TBBL [ $\mu\text{mol}$ of product $\text{min}^{-1}$ ( $\mu\text{L}$ of lysate) $^{-1}$ ]	lysate activity with TBBL, normalized to rabbit PON3	lysate activity with naphthyl acetate [ $\mu\text{mol}$ of product $\text{min}^{-1}$ ( $\mu\text{L}$ of lysate) $^{-1}$ ]	TBBL:naphthyl acetate ratio	lysate activity with paraoxon [ $\text{nmol}$ of product $\text{min}^{-1}$ ( $\mu\text{L}$ of lysate) $^{-1}$ ]	TBBL:paraoxon ratio
rabbit PON3	< 2	1.56	1	0.027	58	0.7	> 230 <sup>a</sup>
round 2 3/6C	~40	38.4	25	0.929	41	3	12700
round 2 5/3E	~40	35.6	23	0.849	42	5.1	6930
round 2 3/6C without trx	< 5	2.68	2	0.025	106	0.5	5770
round 2 5/3E without trx	< 5	1.21	1	0.024	50	0.4	2980
round 3 1/12G	5–10	12.2	8	1.166	11	2.6	4630
round 3 2/12F	5–10	4.1	3	0.142	29	0.7	6020
round 4 1/3C	10	35.0	22	1.877	19	2.3	15380
round 4 5/5D	5–10	23.5	15	0.737	32	3.8	6190
round 5 1/10A	~25	61.7	40	1.598	39	9.8	6310
round 5 3/11B	~15	21.0	14	0.800	26	3.0	6890
round 5 4/4H	~25	58.6	38	1.645	36	9.4	6250

<sup>a</sup> Because of the very low expression levels of wild-type rabbit PON3 and very low paraoxonase activity of PON3, only a threshold for the paraoxonase:lactonase ratio could be determined.

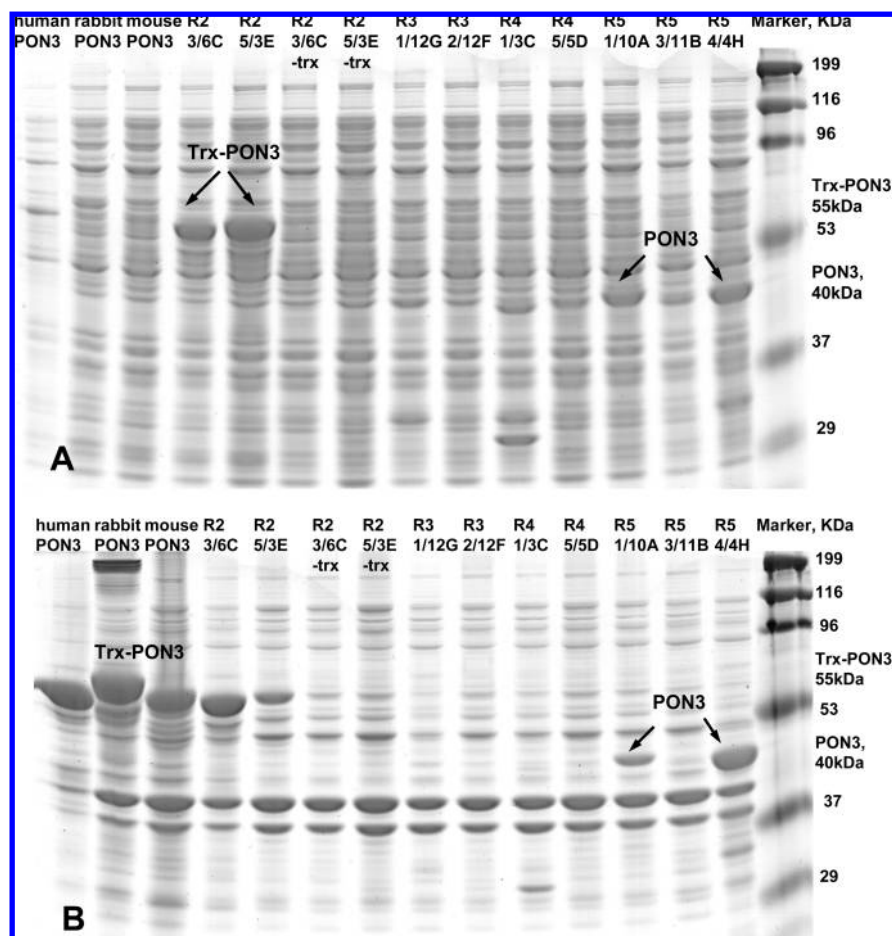


FIGURE 1: SDS-PAGE gels (12%) of crude cell lysates following the overexpression of PON3 variants: (A) supernatant and (B) pellet. Trx-PON3 relates to expression from the pET32b(+) vector expressing PON3 at the C-terminus of thioredoxin. PON3 relates to expression with a His tag at the C-terminus and an intact N-terminus.

4 sequences). It thus appeared unlikely that another round of shuffling would yield variants with high expression levels and stabilities. Clearly, more diversity was needed beyond the three PON3 genes utilized, although our attempts to clone additional PON3s had failed. Analysis of the PON1 variants that had been evolved for bacterial expression indicated that family shuffling had led, in most cases, to the fixation of ancestor/consensus mutations (Table 3 of the Supporting Information); hence, the incorporation of such mutations seemed like a feasible route.

The ancestor/consensus mutations were selected on the basis of a phylogenetic analysis of the PON family and the prediction of the ancestors of the mammalian PON family, including the PON3 subfamily (Supporting Information). The sequences of the evolved round 4 variants were aligned with nine mammalian PON3 sequences and with predicted ancestors (Figures 1 and 2 and Table 4 of the Supporting Information). Several positions were identified at which the evolved PON3 variants had amino acids that differed from the PON3 family consensus (Table 2, residues 3, 8, 188, 257, and 340). Several other positions at which the sequence of the evolved PON3 deviated from the predicted ancestor of mammalian PON3s or from the ancestor of all the mammalian PONs were also probed (Table 2, residues 31, 84, 101, 104, 109, 275, and 280). By homology modeling based on PON1's structure, we ensured that, as is the case for most consensus mutations (18), the ancestor/consensus mutations that we tested were of surface residues (except for residue 340, which is relatively buried) (30), which are also remote from the active site,

so that changes in enzymatic parameters are avoided (Figure 3 of the Supporting Information). The round 5 libraries were created by including the selected consensus/ancestor mutations (12 in total) in the gene shuffling, so that they were incorporated in a combinatorial manner (23). Sequencing of six randomly selected clones from the library indicated an average of  $3 \pm 1$  ancestor/mutation mutations per gene, with compositions of mutations that differ from one gene to another.

Screening of the round 5 library yielded several variants with much improved expression (Figure 1), and stability that allowed their facile purification, concentration, and storage. Round 5 variants 1/10A and 3/11B (dubbed rePON3-1/10A and -3/11B, respectively) were selected for subsequent characterization, on the basis of their highest expression levels, stability (see below), and weak tendency to aggregate.

Soluble expression in *E. coli* generally correlates with configurational stability (kinetic and thermodynamic stability) of the expressed proteins (31). To assess changes in stability, we determined the degree of irreversible thermal inactivation by measuring residual PON3 activity after heating the samples at 50, 60, and 70 °C for 15 min (Figure 4 of the Supporting Information). The evolved variants were significantly more stable than wild-type rabbit PON3, and round 5 variants exhibited the highest stability (they were the only ones that retained some residual activity after being heated at 70 °C). However, tight correlation between the measured stability of evolved PON3 variants and their levels of soluble expression was not observed.

Table 2: Ancestor/Consensus Mutations

residue	residues found in PON3 variants (rounds 2–4)	consensus/ancestor mutation <sup>a</sup>	occurrence in round 5 variants
3	K/N/Q	K (PON3 family consensus and PON3 and PON ancestors)	5 of 6
8	V	T (PON3 family consensus and PON3 and PON ancestors)	6 of 6
31	S/V	F (PON3 and PON ancestors)	2 of 6
84	K	Q (PON3 ancestor)	0 of 6
101	E	N (PON3 ancestor)	0 of 6
104	G/N	D (PON3 ancestor)	0 of 6
109	E	A (PON3 and PON ancestors)	1 of 6
188	S/P	F (PON3 family consensus and PON3 and PON ancestors)	1 of 6
257	E	Q (PON3 family consensus and PON3 and PON ancestors)	0 of 6
275	A	D (PON3 and PON ancestors)	0 of 6
280	L	W (PON family consensus and PON3 and PON ancestors)	4 of 6
340	I	L (PON3 family consensus and PON3 and PON ancestors)	3 of 6

<sup>a</sup> Additional information concerning PON sequence alignments, the phylogenetic tree, and ancestor reconstruction can be found in the Supporting Information.

Table 3: Sequence Characteristics of the Round 5 Variants

	1/10A	3/11B	4/4H	1/1E	1/6F	1/5A
amino acid identity to human PON3 (%)	85	87	87	87	86	89
amino acid identity to rabbit PON3 (%)	91	90	90	92	90	90
amino acid identity to mouse PON3 (%)	79	79	82	81	82	83
ancestor/consensus mutations	T8, F31, W280	T8, A109, W280	T8, F188, W280, L340	T8, L340	T8, F31, W280, L340	T8
random mutations <sup>a</sup>	V6, P188, P240	V6, P188, K211, N230	V6, N250	V6, P188	V6, P188, K296	V6

<sup>a</sup> The random mutations found in round 5 variants correspond to other PON family members: V6 is found in dog PON3 and macaca and taurus PON1; P188 is found in macaca PON3 and is also the consensus residue for PON1 and PON2; K211 is found in mouse PON1; N230 is found in zebrafish PON2 (Figure 1 of the Supporting Information).

For example, variant 3/11B from round 5 is more stable than variant 1/10A from round 5 (Figure 4 of the Supporting Information), but its level of soluble expression is lower (Figure 1). Obviously, other factors play a role in determining expression, such as the stability of the apoenzyme (calcium-free) that must be maintained within *E. coli* cells in which calcium levels are inherently low (32).

**Sequence Composition of the Evolved PON3 Variants.** The evolved PON3 variants were mostly comprised of the parental PON3 rabbit and human genes, with only small insertions taken from the mouse gene (Figure 1 of the Supporting Information). The round 5 variants exhibited 94–97% identity between them, indicating a significant level of convergence by the fifth round. They are 90–92% identical to rabbit PON3, 85–89% identical to human PON3, and only 79–83% identical to mouse PON3. They also carry 1–4 ( $3 \pm 1$ ) ancestor/consensus mutations from the spiked repertoire and 1–4 mutations that were spontaneously incorporated in the course of PCR (Table 3). The latter correspond to residues found in other PON family members and therefore occurred in nonconserved or partially conserved positions. Some of these random mutations appear in more than one variant and actually comprise consensus mutations [e.g., P188 (footnote of Table 3)].

**Enzymatic Characterization of the Evolved PON3s.** RePON3 variants 1/10A and 3/11B were purified, and their activity with various substrates was determined (Table 4). Wild-type rabbit PON3 with a fused thioredoxin tag was used as a reference, and since its expression and stability are marginal, we could obtain only a partially purified sample, which permitted

determination of ratios of activities for various substrates. Specific activity units of the rePON3 variants were compared to those previously reported for human PON3 (1) and were found to be similar, but not identical. These differences appear to stem primarily from differences in expression systems (insect cells for human PON3 vs *E. coli* for rePON3), and detergent, rather than from differences in sequence or in active site structures. Indeed, the evolved PON3 variants do not bear mutations in the active site or close to it. Lipids and detergents have significant effects on the enzymatic activity of PON1 and, presumably, also on that of PON3. Differences in the size and type of micelles, or of lipid particles, to which the enzyme is anchored (Table 2 of the Supporting Information) may affect the active site conformation. Indeed, the same variant (1/10A) tested in two different detergents (tergitol and zwittergent) shows 3–6-fold variations with lactone and ester substrates.

The ratios of activities with various substrates compare well with those of rabbit and human PON3, indicating that both the lactonase and promiscuous activities of the rePON3 variants were not significantly altered by the directed evolution process.

As previously reported for human PON3, rePON3 variants exhibited the highest activity toward lactones. The catalytic parameters for the lactones are similar for the two rePON3 variants, with variant 1/10A being 2–3-fold more active (Table 5). The activity is highest for TBBL, the substrate for which the PON3 variants were screened in the course of directed evolution. Overall, the catalytic parameters of PON3 (both rePON3 and human PON3) with aliphatic lactones are of the same order of magnitude

Table 4: Specific Activities of the Evolved PON3 Variants from Round 5 (units of micromoles per minute per milligram of enzyme)

	human PON3, <sup>a</sup> dodecyl maltoside	rabbit PON3, <sup>c</sup> tergitol	rePON3-1/10A, tergitol	rePON3-1/10A, zwittergent	rePON3-3/11B, zwittergent
$\gamma$ -nonanoic lactone, 1 mM	30.9 (100% <sup>b</sup> )	100%	40.7 (100%)	27.6 $\pm$ 0.8 (100%)	17.8 $\pm$ 1.7 (100%)
$\gamma$ -butyrolactone, 1 mM	0.81 (2.6%)	not measured	not measured	below threshold	below threshold
$\gamma$ -caprolactone, 1 mM	23.9 (77%)	not measured	not measured	2.4 $\pm$ 0.3 (8.7%)	1.53 $\pm$ 0.04 (8.6%)
$\gamma$ -heptanamide, 1 mM	27.7 (89.6%)	not measured	not measured	10.2 $\pm$ 1.7 (36.9%)	4.9 $\pm$ 0.2 (27.5%)
$\gamma$ -undecanoic lactone, 0.5 mM	35.7 <sup>c</sup> (115%)	114%	72.7 (178%)	28 $\pm$ 2 (101%)	22.5 $\pm$ 2.4 (126%)
$\gamma$ -dodecanoic lactone, 0.5 mM	not measured	not measured	not measured	5.0 $\pm$ 0.6 (18.1%)	18.5 $\pm$ 2.4 (104%)
$\delta$ -nonanoic lactone, 1 mM	11.1 (35.9%)	not measured	not measured	72 $\pm$ 8 (261%)	15.0 $\pm$ 0.9 (84%)
$\delta$ -undecanoic lactone, 0.5 mM	42.2 <sup>c</sup> (136%)	not measured	not measured	32.1 $\pm$ 1.8 (116%)	24 $\pm$ 1 (135%)
$\delta$ -dodecanoic lactone, 0.5 mM	not measured	not measured	not measured	54 $\pm$ 2 (196%)	42 $\pm$ 5 (239%)
dihydrocoumarin, 0.2 mM	126.1 (408%)	70.8%	152 (373%)	93 $\pm$ 11 (337%)	74 $\pm$ 16 (416%)
2-coumarone, 0.3 mM	20.3 <sup>c</sup> (66%)	not measured	not measured	27 $\pm$ 4 (97.8%)	10.7 $\pm$ 1.3 (60.1%)
TEBL, 0.22 mM	not measured	26.5%	2.46 (6%)	7 $\pm$ 1 (25.4%)	7.3 $\pm$ 0.6 (41%)
TBBL, 0.4 mM	not measured	114%	30.8 (76%)	96 $\pm$ 10 (348%)	68 $\pm$ 5 (382%)
THBL, 0.114 mM	not measured	not measured	not measured	64 $\pm$ 10 (232%)	34.5 $\pm$ 0.5 (194%)
lovastatin, 0.2 mM	0.27 (with 0.025 mM)	not measured	not measured	3.1 $\pm$ 1.1	6.5 $\pm$ 0.2
estradiol 3,17-diacetate, 0.1 mM	55.4 (with 10 $\mu$ M) <sup>d</sup>	not measured	not measured	13.3 $\pm$ 0.1	8.6 $\pm$ 0.2
naphthyl acetate, 0.2 mM	not measured	not measured	1.49 (3.7%)	8.6 $\pm$ 1.0 (31.1%)	8.4 $\pm$ 0.1 (47.2%)
phenyl acetate, 1 mM	4.1 (13.3%)	below detection limit	below detection limit	2.81 $\pm$ 0.08 (10.2%)	1.13 $\pm$ 0.02 (6.3%)
p-NO <sub>2</sub> phenyl acetate, 0.5 mM	19.5 <sup>c</sup> (63%)	1%	1.73 (4.2%)	10.4 $\pm$ 0.4 (37.7%)	8.9 $\pm$ 0.4 (50%)
p-NO <sub>2</sub> phenyl butyrate, 0.5 mM	5.7 (18.5%)	not measured	not measured	2.71 $\pm$ 0.08 (9.8%)	4.50 $\pm$ 0.05 (25.3%)
p-NO <sub>2</sub> phenyl octanoate, 0.25 mM	not measured	not measured	not measured	4.4 $\pm$ 0.3 (15.9%)	7.7 $\pm$ 0.2 (43.2%)
paraoxon, 1 mM	0.205 (0.66%)	below detection limit	0.0061 (0.015%)	0.0164 $\pm$ 0.0001 (0.06%)	0.0229 $\pm$ 0.0007 (0.13%)

<sup>a</sup> Taken from ref (1). <sup>b</sup> PON3 activities with various substrates were normalized to the activity with  $\gamma$ -nonanoic lactone, which was assigned as 100%. <sup>c</sup> The activities were originally determined with 1 mM substrate. <sup>d</sup> Taken from ref (25). <sup>e</sup> The expression levels of rabbit PON3 were very low (< 2 mg/L of culture) and unstable (many productions resulted in almost no active enzyme), and purification resulted in only ~30% pure enzyme as judged by SDS-PAGE. The ratios between the various activities of rabbit PON3 were therefore taken as a reference, and not their absolute values.

Table 5: Catalytic Parameters of the rePON3 and rePON1 Variants with Lactones

	TBBL	$\gamma$ -nonanoic lactone	$\gamma$ -undecanoic lactone	$\delta$ -undecanoic lactone
PON3 round 5 1/10A	$k_{\text{cat}}$ (s <sup>-1</sup> )	42 $\pm$ 8	22 $\pm$ 1	21 $\pm$ 1
	$K_{\text{M}}$ (mM)	0.44 $\pm$ 0.14	1.1 $\pm$ 0.1	0.47 $\pm$ 0.03
	$k_{\text{cat}}/K_{\text{M}}$ (s <sup>-1</sup> M <sup>-1</sup> )	99200 $\pm$ 11400	20550 $\pm$ 1850	43700 $\pm$ 1270
PON3 round 5 3/11B	$k_{\text{cat}}$ (s <sup>-1</sup> )	14 $\pm$ 2	7.0 $\pm$ 0.5	10 $\pm$ 2
	$K_{\text{M}}$ (mM)	0.33 $\pm$ 0.07	1.1 $\pm$ 0.1	0.6 $\pm$ 1
	$k_{\text{cat}}/K_{\text{M}}$ (s <sup>-1</sup> M <sup>-1</sup> )	41400 $\pm$ 2400	6240 $\pm$ 640	18000 $\pm$ 980
rePON1 <sup>a</sup>	$k_{\text{cat}}$ (s <sup>-1</sup> )	116 $\pm$ 4	31 $\pm$ 2	62 $\pm$ 2
	$K_{\text{M}}$ (mM)	0.27 $\pm$ 0.04	0.39 $\pm$ 0.03	0.60 $\pm$ 0.07
	$k_{\text{cat}}/K_{\text{M}}$ (s <sup>-1</sup> M <sup>-1</sup> )	440000 $\pm$ 55000	78000 $\pm$ 1600	103000 $\pm$ 8600

<sup>a</sup> Catalytic parameters are provided for recombinant PON1 variant P2E6, that exhibits wild-type like enzymatic specificity (2, 11). <sup>b</sup> The catalytic parameters of rePON1 were not measured with  $\delta$ -undecanoic lactone.

as those of PON1 ( $k_{\text{cat}}/K_{\text{M}} \sim 10^5 \text{ s}^{-1} \text{ M}^{-1}$ ), although the rates exhibited by PON1 are 2–5-fold higher.

The esterase activity of the rePON3 variants is much lower than their lactonase activity (2–25-fold, depending on the substrate and on the detergent), and the phosphotriesterase activity was barely detectable, which was the case with human and rabbit PON3. The rePON3 variants also hydrolyze lovastatin and 3,17-estradiol diacetate, which had previously been assigned as exclusive substrates of human PON3 (with PON1 and PON2 displaying little or no activity toward them) (9, 25).

Overall, the sequence and enzymatic parameters seem to indicate that the rePON3 variants reproduce the properties of the wild-type rabbit and human PON3s, with the major differences being the high stability and bacterial expression of the rePON3s.

**PON3–HDL Interactions.** It is known that PON1 is a HDL-associated protein, and the interaction of recombinant

PON1 with reconstituted HDL has been characterized in detail (26, 33, 34). A substantial fraction of PON3 is HDL-associated in rabbit (35) and human serum (36), but its interactions with HDL have not been studied before. The rePON3 variants provided an opportunity to examine the HDL-related properties of PON3. We examined the interaction of PON3 with apoAI-HDL in particular [using reconstituted HDL particles based on recombinant apoAI (rHDL particles)] and tested whether the stabilization and stimulation effects were similar to those observed for PON1 (26).

**Stabilization of PON3 by rHDL.** To analyze the effect of rHDL on rePON3 stability, the rate of enzyme inactivation was monitored in the presence and absence of rHDL. Delipidated rePON3-1/10A and -3/11B in buffer, or in rHDL, were incubated in the presence of the calcium chelator (EDTA) to expedite inactivation due to loss of PON3's calcium ions, and the residual lactonase activity was measured as a function of time.

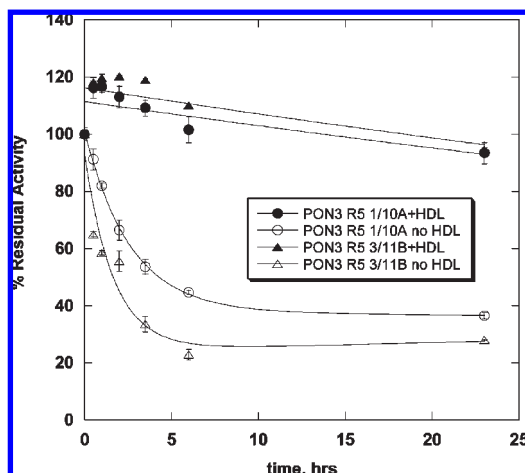


FIGURE 2: Kinetics of the inactivation of rePON3 variants in the absence and presence of rHDL particles. Delipidated rePON3 variants ( $0.4 \mu\text{M}$ ) were incubated with an excess of rHDL ( $10 \mu\text{M}$ ) or activity buffer and subjected to inactivation by EDTA ( $2 \text{ mM}$ ) at  $37^\circ\text{C}$ . Aliquots were taken at suitable time points, and the residual activity was measured with TBBL ( $0.25 \text{ mM}$ ). The initial activity of the delipidated enzyme is taken to be 100%.

The residual activity was compared to the initial activity of the delipidated enzymes (Figure 2). In the presence of rHDL, rePON3 lost 20% of its initial activity within 20 h. In the absence of the rHDL, inactivation occurred much faster, rePON3 lost 80% of its activity within 10 h, with a rate similar to that observed for rePON1 (26). For rePON3 bound to rHDL particles, the inactivation profiles fitted a monoexponential regime ( $t_{1/2}^{\text{inact}} \sim 84 \text{ h}$  for rePON3-1/10A and  $\sim 87 \text{ h}$  for rePON3-3/11B), indicating the involvement of a single rePON3–HDL complex. However, in the absence of rHDL, inactivation followed a double-exponential regime. The fast inactivation step is associated with a much less stable form of PON3 ( $t_{1/2}^{\text{inact}} \sim 1.8 \text{ h}$  for rePON3-1/10A, and  $t_{1/2}^{\text{inact}} \sim 1.1 \text{ h}$  for rePON3-3/11B), whereas the slow phase is associated with a much more stable species, displaying a rate of inactivation similar to that displayed by the rePON3–HDL complex. Overall, the inactivation profiles obtained for rePON3 are similar to those measured for rePON1 (26), with similar inactivation half-lives both in the presence and in the absence of rHDL ( $t_{1/2}^{\text{inact}} \sim 70$  and  $0.2 \text{ h}$ , respectively).

**Stimulation of Lactonase Activity by rHDL.** Delipidated PON3 variants 1/10A and 3/11B were incubated with various concentrations of rHDL particles (corresponding to HDL:PON ratios of 0–7.5), and the resulting enzymatic activity was measured with aliphatic lactones ( $\gamma$ -undecanoic lactone and TBBL) and an aromatic ester [*p*-nitrophenyl acetate (Figure 3 and Figure 5 of the Supporting Information)]. To confirm that activity stimulation is specific to apoAI-HDL, the delipidated enzymes were also incubated with increasing concentrations of detergent (zwittergent). Once rHDL bound, the lactonase activity of the rePON3 variants with  $\gamma$ -undecanoic lactone increased by 8–12-fold. Incubation with detergent led to more modest stimulations ( $\leq 4$ -fold). These results are similar to stimulation of rePON1 lactonase activity that increased 13–20-fold upon binding to apoAI-rHDL particles (26). The arylesterase activity (*p*-nitrophenyl acetate hydrolysis) and the activity with TBBL were stimulated much less [ $< 4$ -fold activity increase (Figure 5 of the Supporting Information)], and the effect was similar to that obtained with detergent (either zwittergent or tergitol, 2–4-fold activity increase). In the case of rePON1, the promiscuous

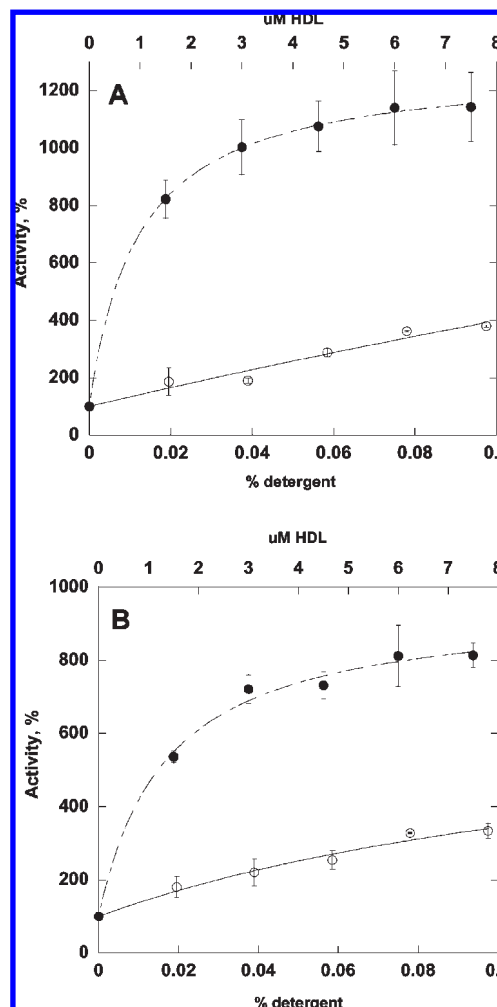


FIGURE 3: Stimulation of the lactonase activity of rePON3 variants by rHDL. Delipidated rePON3 variants [(A) rePON3-1/10A and (B) rePON3-3/11B] were incubated with increasing concentrations of rHDL particles (●) or zwittergent (○), and rates of lactonase activity were determined with  $\gamma$ -undecanoic lactone ( $0.5 \text{ mM}$ ). Activity is presented as the percentage of the initial activity of the delipidated enzyme.

esterase and paraoxonase activities were also stimulated much less than lactonase activity ( $\leq 5$ -fold) (26). The activity of both rePON3 variants tested was similarly stimulated, the major difference being that rePON3-3/11B was more sensitive to delipidation, and harsh delipidation conditions which could be used for rePON1 and rePON3 variant 1/10A led to complete activity loss in rePON3-3/11B.

**The Anti-Atherogenic Potential of PON3.** (i) *Macrophage-Mediated LDL Oxidation.* Various studies connect PON1 and PON3 to prevention of atherosclerosis (7, 8, 35, 37, 38). These studies include transgenic mice with PON1 and PON3 knockouts and overexpression. The anti-atherogenic potential of human PON1 is well-studied, and that of rePON1 seems comparable, if not favorable [especially in terms of stability, and under conditions that mimic oxidative stress (L. Gaidukov et al., submitted for publication)]. Specifically, PON1 has been shown to inhibit cell-mediated LDL oxidation and to enhance HDL-mediated cholesterol efflux from macrophages, in ex vivo tests (27). Certain studies suggest that the anti-atherogenic potential of PON3 is comparable, or even higher (6, 7, 35), but such ex vivo tests have not been performed with PON3. To determine the ability of rePON3 to inhibit macrophage-mediated

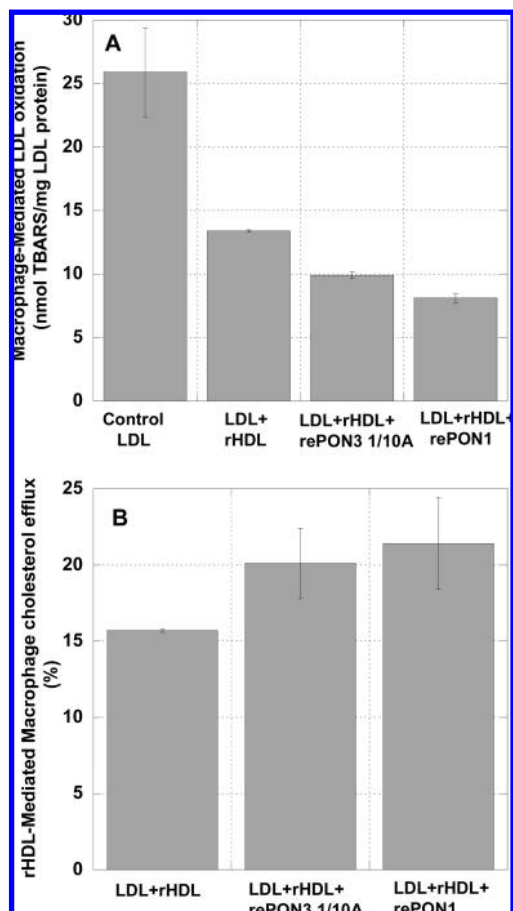


FIGURE 4: Effects of rHDL-associated rePON3 on macrophage-mediated LDL oxidation (A) and on rHDL-mediated cholesterol efflux from macrophages (B).

LDL oxidation, LDL was incubated with J774A.1 macrophages in the presence of copper ions and in the presence of either the rePON3–rHDL or rePON1–rHDL complex. As a reference, LDL was incubated under the same conditions without the addition of rePON samples. The extent of LDL oxidation was then measured with the TBARS assay (28). The addition of rHDL alone inhibited LDL oxidation by the cells by 48%, from  $25.9 \pm 3.5$  nmol of TBARS/mg of LDL protein for the control containing LDL alone to  $13.4 \pm 0.8$  nmol/mg of LDL protein in the sample containing LDL and rHDL (Figure 4A). When rePON3 variant 1/10A was associated with rHDL, the inhibitory effect of rHDL increased by 26% (from  $13.4 \pm 0.8$  to  $9.9 \pm 0.3$  nmol of TBARS/mg of LDL protein). The level of inhibition by the rePON1–rHDL complex was slightly higher than that of the rePON3–rHDL complex ( $8.1 \pm 0.4$  nmol of TBARS/mg of LDL protein; 39% more inhibition relative to that produced by rHDL alone).

(ii) *rHDL-Mediated Cholesterol Efflux from Macrophages.* The ability of rePON3 to enhance HDL-mediated cholesterol efflux from macrophages was studied by incubating rHDL-associated rePON3 variant 1/10A with macrophages containing [ $^3$ H]cholesterol. As shown in Figure 4B, and in our previous studies, association of rePON1 with rHDL enhanced the ability of rHDL to induce cholesterol efflux from J774A.1 macrophages by 36% (from  $15.7 \pm 0.1$  to  $21.4 \pm 3.0\%$ ). Similarly, but to a lesser extent, rePON3 associated with rHDL stimulated the cholesterol efflux rate by 28% as compared to rHDL on its own (from  $15.7 \pm 0.1$  to  $20.1 \pm 2.3\%$ ).

## DISCUSSION

*Bacterial Expression by Family Shuffling and Phylogeny-Based Mutagenesis.* The aim of this evolutionary process was to provide an ample source of recombinant PON3 by expression in *E. coli*. This was achieved by modification of the previous evolutionary experiments performed on PON3 (4), in which an increase in the level of bacterial expression was accompanied by large changes in enzymatic specificity. In this study, libraries were screened for lactonase activity, and the ratios between the lactonase activity and the promiscuous esterase and phosphotriesterase activities were monitored throughout. It was thus possible to obtain evolved PON3 variants that are enzymatically similar to wild-type rabbit and human PON3s but express in a soluble and functional manner in *E. coli*. In the course of this directed evolution process, it was found that PON3's promiscuous activities are highly evolvable, as are the promiscuous activities of many other enzymes (39). The promiscuous esterase and phosphotriesterase activities were both easily enhanced, even though no selection for such improvements had been applied.

A unique aspect of this work relates to the combination of the traditional family shuffling procedure (13, 40), with phylogeny-based mutagenesis, and with ancestor mutations in particular. The consensus method is based on sequence alignment of homologous genes and on modification of residues differing from the consensus. It has been utilized for stabilization of numerous proteins (16, 18, 19, 41). In addition to consensus mutations, ancestral mutations have been used here in an attempt to improve protein stability, the underlying assumption being that the ancestors were more thermostable than contemporary proteins (20–22). Ancestor mutations are not routinely applied for protein engineering, and in most positions, they overlap the consensus (Table 2). However, there are stabilizing mutations that appear uniquely in the ancestor analysis yet prove to be beneficial [e.g., F31 and A109 (Table 3)] (21, 42). In effect, the consensus mutations complement the family shuffling by introducing diversity that could come from family members that could not be cloned and were therefore not represented in the shuffling mutations. Indeed, consensus mutations underlined the outcome of family shuffling and selection of PON1 for increased stability and bacterial expression (Table 3 of the Supporting Information). The ancestor mutations, on the other hand, represent diversity that could not be sampled by family shuffling, because the cDNAs of the genes were not available. The incorporated ancestor mutations in most of the cases were present in the nonavailable genes [e.g. dog or pig (Figure 1 of the Supporting Information)]. Both consensus and ancestor mutations were incorporated into the selected PON3 variants (Tables 2 and 3), leading to higher levels of soluble expression and stability. These mutations are also valuable as they may act as global suppressors, thus increasing tolerance of mutations and promoting the evolution of new enzyme functions (42, 43). Hence, libraries based on gene shuffling, with spiking of phylogeny-based mutations, provide a general way of generating stable, evolvable protein variants of human, and presumably other, proteins that exhibit limited stability and ineffective bacterial expression.

*PON3–HDL Interactions and Anti-Atherogenic Potential.* The sequence and enzymatic parameters seem to indicate that the rePON3 variants reproduce the properties of the wild-type rabbit and human PON3s and thus provide a reliable and convenient model for studying the biochemical properties of PON3. Analysis of the rHDL interactions indicates that PON3

interacts with apoAI-HDL particles in a manner similar to that of PON1 (26). The stability of rePON3 increased markedly when it was bound to rHDL (half-life in the presence of calcium chelating agent increases by >45-fold). The lactonase activity of rePON3 was stimulated upon binding to rHDL by ~10-fold, and significantly more than upon detergent addition. These results indicate that, as found for PON1, the interaction with apoAI-HDL is specific and induces changes in the enzyme's active site.

PON3 was also found to exhibit anti-atherogenic properties. When associated with rHDL, rePON3 inhibited LDL oxidation and stimulated the HDL-mediated cholesterol efflux from macrophages, albeit to a lesser degree than PON1.

Structural studies based on rePON3-1/10A are currently in progress. A structure of PON3 not only would stand for itself but also would provide insights into the differences between the specificities of PON1 and PON3.

### SUPPORTING INFORMATION AVAILABLE

List of primers used in this work, description of detergent choice, multiple-sequence alignments and description of ancestor reconstruction, a model of the three-dimensional PON3 structure with the phylogenetic mutations, and the results of the thermal inactivation experiment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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