

Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization

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Serum paraoxonases (PONs) are a group of enzymes that play a key role in organophosphate (OP) detoxification and in prevention of atherosclerosis. However, their structure and mechanism of action are poorly understood. PONs seem like jacks-of-all-trades, acting on a very wide range of substrates, most of which are of no physiological relevance. Family shuffling and screening lead to the first PON variants that express in a soluble and active form in *Escherichia coli*. We describe variants with kinetic parameters similar to those reported for PONs purified from sera and others that show dramatically increased activities. In particular, we have evolved PON1 variants with OP-hydrolyzing activities 40-fold higher than wild type and a specificity switch of >2,000-fold, producing PONs specialized for OP rather than ester hydrolysis. Analysis of the newly evolved variants provides insights into the evolutionary relationships between different family members.

The paraoxonases (PONs) are a family of closely related enzymes, which include PON1, PON2, and PON3 and share 60–70% nucleic acid identity. The two PON1 isoenzymes Q and R are by far the most investigated family members. They are calcium-dependent hydrolases that catalyze the hydrolysis of a broad range of esters and lactones (1, 2). PON1s also catalyze, albeit at much lower rates, the hydrolysis and thereby inactivation of various organophosphates (OPs), including the nerve agents sarin and soman (3). Initial interest in PONs was, therefore, toxicological. In addition, PON1 is involved in drug metabolism and is being used for drug inactivation (1, 4). In recent years, it has become apparent that PONs play an important role in the prevention of atherosclerosis. PON1 and PON3 reside in the cholesterol-carrying particles high-density lipoprotein (HDL, “good” cholesterol). The levels of PON1 in the blood and its catalytic proficiency appear to have a major impact on the individual’s susceptibility to pollutants and insecticides and to atherosclerosis (5). Furthermore, mice lacking the PON1 gene are highly susceptible to atherosclerosis and to OP poisoning (6). *In vitro* assays show that PON1 inhibits the oxidation of lipids in low-density lipoprotein (LDL, “bad” cholesterol), thereby reducing the level of oxidized lipid forms involved in the initiation and progression of atherosclerosis (7–9). Because atherosclerosis is the underlying cause of ≈50% of all deaths in Western societies, and because nerve gases are both a terrorist threat and a nonconventional military weapon, PONs are currently the subject of intensive research.

Despite many efforts, the structure and mechanism of action of PONs are still enigmatic. First, PONs hydrolyze a broad repertoire of substrates, ranging from phosphotriesters to esters (of carboxylic acids) and lactones (1, 2). Roles in the hydrolysis of platelet-activating factor (10) and of L-homocysteine thiolactone (L-HcyT, a known risk factor for atherosclerotic vascular disease) (2) and in reduction of lipid peroxides (9) have also been suggested. However, these activities of PON1 are orders of magnitude lower than with its well characterized substrates. For example, hydrolysis of L-HcyT is 2,800–25,000 times lower than hydrolysis of phenylacetate, depending on the enzyme preparation (2, 11). Such low and varying activities may be due to

miniscule amounts of contaminating enzymes. Indeed, a recent article argues that platelet-activating factor hydrolysis is due to the presence of low levels of platelet-activating factor acetyl hydrolase (12). PON3’s low level in the high-density lipoprotein (≈50-fold lower than PON1) hinders its purification and detailed characterization. PON3s were only recently purified and characterized from human and rabbit sera (13, 14). They were found to hydrolyze a wide variety of esters and lactones but not paraoxon. PON3 appears to exhibit enhanced ability to protect low-density lipoprotein against oxidation relative to PON1, suggesting a key role in prevention of atherosclerosis (13).

Access to recombinant PONs would afford several benefits. It would greatly facilitate their structural and functional characterization and permit examination of their weak, yet potentially most biologically relevant, activities, in the complete absence of other serum enzymes. PONs constitute a mystery: the best known substrates (e.g., phenylacetate and dihydrocoumarin) are man-made and have no known physiological relevance, whereas the enzymes perform very poorly with substrates of potential biological significance, such as L-HcyT and oxidized lipid esters. Nor is PON1 a highly effective phosphotriesterase. Its catalytic specificity (k_{cat}/K_M) for paraoxon is >200-fold lower than for phenylacetate and needs to be significantly improved for effective treatment of OP detoxification (15). PONs therefore constitute an intriguing target for engineering aimed at improving catalytic efficiency and specificity. Perhaps these “enzymes of many substrates” could be compelled to “specialize” for both practical applications and mechanistic studies. Such engineering entirely depends on the ability to amply express and screen many thousands of variants. It was shown, however, that PON1 could not be functionally expressed in *Escherichia coli*, presumably because of aggregation and the absence of glycosylation (16, 17).

Directed evolution is a powerful tool for reshaping functional and structural features of proteins, including their solubility (18) and their enzymatic proficiency and specificity (19, 20). Here we report application of directed evolution in achieving functional expression of PON1 and PON3 in *E. coli* at >20 mg/liter culture and a dramatic increase in their hydrolytic proficiency toward the fluorogenic OP substrate 7-*O*-diethylphosphoryl-3-cyano-7-hydroxycoumarin (DEPCyC).

Materials and Methods

Further experimental details are provided as *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Abbreviations: PON, paraoxonase; OP, organophosphate; DEPCyC, 7-*O*-diethylphosphoryl-3-cyano-7-hydroxycoumarin; 2NA, 2-naphthylacetate; L-HcyT, L-homocysteine thiolactone.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY499188–AY499199).

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Cloning of PON Genes and Construction of Gene Libraries. Plasmids containing the HuPON1 and HuPON3 genes (14) were used as templates for PCR amplification. The genes for MoPON1, MoPON3, and RatPON1 were amplified from mouse and rat liver cDNA (Clontech). RabPON1 and RabPON3 were amplified from cDNA prepared from fresh liver of New Zealand rabbits. All genes were cloned into pET32b(+) (Novagen). Primers used for amplification are listed in Table 4, which is published as supporting information on the PNAS web site. For the construction of PON1 and PON3 libraries by DNA shuffling, the PON genes were individually amplified, mixed in equal amounts, digested, and shuffled as described (21). The resulting PON1 and PON3 gene libraries were recloned into pET32b and transformed into DH5 α cells. DH5 α colonies ($>5 \times 10^4$) were combined, and the plasmid DNA was extracted. Randomly mutagenized PCR libraries were derived from highly soluble rePON1 variant G3C9 genes by using wobble-base PCR (22). Third-generation shuffling and randomly mutagenized PON1 libraries were cloned into a pET32 vector from which the Trx fusion protein and peptide tags had been truncated (pET32-tr).

Screening Procedures. Libraries were transformed into origamiB (DE3, Novagen) cells and grown and replicated with velvet cloth for the esterase screening. A layer of soft agar (0.5%) in activity buffer (50 mM Tris, pH 8/1 mM CaCl₂) supplemented with 0.3 mM 2-naphthylacetate (2NA) and 1.3 mg/ml Fast Red was added (23). Colonies that turned red first were picked from the replica plate. For the OP screen, a layer of soft agar (0.5%) supplemented with 40 μ M fluorogenic substrate DEPCyC (for synthesis, see Fig. 4, which is published as supporting information on the PNAS web site) was added to the original plates. Highly fluorescent colonies were picked directly from the original plate. Positive colonies were used to inoculate 500 μ l of LB media in a 96-deep-well plate and grown overnight at 30°C. Plates were duplicated, lysed with BugBuster (Novagen), and screened for 2NA, paraoxon, and DepCyC hydrolysis in 96-well plates. The activity of the best 20 clones was verified, and their plasmid was extracted and used as a template for subsequent rounds of reshuffling.

Expression and Purification of the rePON Variants. Typically, 1 liter of LB medium was inoculated and grown overnight at 30°C. Cells were harvested by centrifugation, resuspended, and disrupted by sonication. Ammonium sulfate was added to the lysate to 55% (wt/vol). The precipitate was dissolved and dialyzed against activity buffer and then chromatographed on a NiNTA column followed by a High Trap Q column (Pharmacia). Fractions were analyzed for paraoxonase activity and purity (by SDS/PAGE), pooled, dialyzed, and concentrated. For purification of rePON1 (without the Trx and affinity tags), the dialyzed ammonium sulfate precipitate was chromatographed on High Trap Q as above, followed by ceramic hydroxyapatite column (Type 1, Bio-Rad).

Enzymes Kinetics. A range of enzyme concentrations (0.005–4 μ M) and substrate concentrations was applied (from $0.3 \times K_M$ up to $2-3 \times K_M$). Product formation was monitored spectrophotometrically in 96-well plates with 200- μ l reaction volumes. Kinetic parameters were determined by fitting the data directly to the Michaelis–Menten $\{V_o = k_{cat}[E]_0[S]_0/([S]_0 + K_M)\}$ and competitive inhibition $\{V_o = V_{max}[S]_0/([S]_0 + K_M(1 + [I]/K_i))\}$ models. Background hydrolysis of paraoxon, *p*-nitrophenylacetate, and L-HcyT and DEPCyC were subtracted from enzyme kinetic experiments.

Results and Discussion

Family DNA Shuffling and Screening. The wild-type PON1 genes, human (R-form; Hu), mouse (Mo), rat (Rat), and rabbit (Rab)

(79–93% nucleic acid sequence identity), and wild-type PON3 genes Hu, Mo, and Rab (81–87% identity), were cloned into an expression vector encoding the PON genes fused to thioredoxin by a peptide linker. Fusion to thioredoxin slightly increased the solubility of PONs (24) and permitted detection of trace activities in lysates of Mo-, Rat-, and Rab-PON1. To achieve efficient expression in *E. coli* and increase the specific activity of PON toward different substrates, we used family DNA shuffling followed by screening for different activities. DNA shuffling of families of homologous genes has proven a powerful technique for directed evolution of enzymes with specific functions (21, 25). We created two separate libraries by DNA shuffling, a PON1 library derived from shuffling of four wild-type PON1 genes and a PON3 library derived from three wild-type PON3 genes. The libraries were transformed to *E. coli*. Sequencing of five randomly chosen clones from each library indicated >10 crossovers per gene with almost even distribution of the parental genes (Fig. 5, which is published as supporting information on the PNAS web site). The libraries were screened on agar plates for esterase activity by using 2NA and an azo dye (Fast Red) that reacts with the released 2-naphthol to generate an insoluble red product (23); 10^3 to 10^4 individual agar colonies were screened in each round. Typically, 200–500 clones exhibiting the highest rate of color formation were picked from a replica plate and grown in liquid medium; the crude cell lysate was assayed spectrophotometrically for the hydrolysis of various substrates, including paraoxon and 2NA. The 20 best clones were subcloned and used as templates for further rounds of DNA shuffling and screening.

Directed Evolution of PON1 for *E. coli* Expression. After three rounds of shuffling and screening, improved clones of PON1 were isolated. The crude lysates of these clones exhibited ≈ 20 -fold higher rates with both 2-NA and paraoxon. Further analysis indicated increased levels of soluble, active enzyme rather than increased specific activity. The best first-generation variants were expressed fused to thioredoxin tag (trx-rePON1 variants G1A5 and G1C4) and exhibited a 3- to 6-fold increase in the amount of soluble protein relative to trx-RabPON1. The best clones from the second round of evolution (G2D6 and G2E6) exhibited a 14- to 18-fold increase (Fig. 1). We then recloned the second-generation library to an expression vector without the fusion protein (thioredoxin) and linker peptide, retaining the unmodified, 355-aa PON1 proteins (rePON1). Screening of this library generated variants G3C9 and G3H8, which yielded ≈ 12 mg/liter culture of pure, active, unmodified PON1 (Fig. 6, which is published as supporting information on the PNAS web site). These newly evolved variants stand in clear contrast to the unmodified wild-type PON1s, including RabPON1, that yield no soluble expression without the thioredoxin tag.

The sequences of the selected variants reveal that, as early as the first round of evolution, convergence to the RabPON1 gene has occurred, with relatively small contributions from the other three parental genes (Fig. 7A, which is published as supporting information on the PNAS web site). The convergence toward RabPON1 is not surprising, given that the wild-type RabPON1 was the only wild-type PON1 that, after fusion to thioredoxin, expressed measurable amounts of soluble enzyme in *E. coli* (Fig. 1). It may be related to the higher stability of serum RabPON1 (certainly relative to HuPON1) and its higher affinity for calcium (26). In all selected variants, position 192 is a lysine as in the rabbit wild-type sequence. This position is either an arginine or asparagine in the human R/Q PON1 isoenzymes, of which the R form exhibits ≈ 8 -fold higher catalytic activity (27). Lysine is probably equivalent to arginine, hence the resemblance of the rate parameters of the new variants to the R form human PON1 (Table 1). Eight conserved mutations were identified in all the selected variants, located in only two regions, residues 126–142 and 301–343 (Fig. 8, which is published as supporting informa-

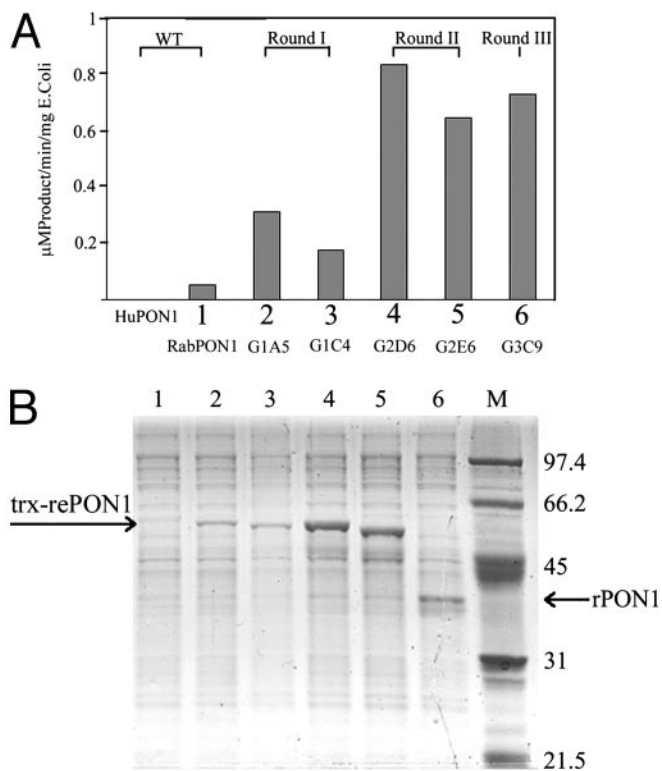


Fig. 1. Directed evolution of PON1 for soluble expression in *E. coli*. (A) Paraoxonase activity of PON1 variants in the crude *E. coli* lysate calculated per milligram of *E. coli* cells. Activity is shown for the wild-type trx-HuPON1 (nil), trx-RabPON1, trx-rePON1 variants G1A5 and G1C4 from the first round and G2D6 and G2E6 from the second round, and rePON1 variant G3C9 (devoid of all tags) from the third round of evolution. (B) SDS/PAGE (12%) of the crude cell lysates as numbered in A.

tion on the PNAS web site). The mutations in the first region are I126T, M130L, K137S, and L142V, and in the second region the mutations are A301G, A320V, M341L, and V343I. All the mutations are quite subtle, and half are from one hydrophobic amino acid to another (M130L, L142V, M341L, and V343I). The two mutated regions in the newly evolved PON1s may be involved in correct folding of PON1 and in its oligomeric

packing. It has been suggested that exposed hydrophobic surfaces of PON1 are involved in its oligomerization and aggregation (17). These putative hydrophobic surfaces may also lead to aggregation of wild-type PON1s when expressed in *E. coli*. Subtle mutations in these exposed hydrophobic surfaces may have prevented misfolding and formation of high-order aggregates and thereby facilitated soluble expression.

Selected PON1 variants were purified, and their kinetic parameters were determined (Table 1 and Fig. 9, which is published as supporting information on the PNAS web site). Little variation occurs in the catalytic parameters for paraoxon and phenylacetate hydrolysis between variants of the first, second, or third generations. Other substrates (2NA and dihydrocoumarin) show the same trend (Table 5, which is published as supporting information on the PNAS web site). This suggests that the increased catalytic activity in the isolated clones is mainly due to increased protein solubility and that the enzymatic properties of PON1 were not significantly altered. Detailed kinetic parameters for wild-type RabPON1 (to which the newly evolved clones are most homologous) are not available. However, serum RabPON1 and serum HuPON1 display very similar rates (26), and the newly evolved rePON1s do exhibit kinetic parameters similar to serum HuPON1 (Table 1). The substrate specificity of serum RabPON1 has also been determined (13), and our rePON1s appear quite similar (Table 6, which is published as supporting information on the PNAS web site). Other key properties of PON1 seem to be retained in the newly evolved variants. Like HuPON1 (17), rePON1s appear to be in monomer-dimer equilibrium in the presence of 0.6 mM C₁₂-maltoside, as analyzed by gel filtration (data not shown).

Homocysteine Thiolactonase Activity of rePON1 and Exclusion of Multiple Sites of Hydrolytic Activity. A recent and intriguing finding regarding PON1 is its putative ability to hydrolyze L-HcyT, which is a major risk factor in atherosclerosis (2, 11). We measured such activity in both trx-reRabPON1 and in the newly evolved variants (e.g., G1A5 and G3H8; Table 5 and Fig. 10, which are published as supporting information on the PNAS web site). The K_M measured for L-HcyT hydrolysis (≈ 19 mM) is in good agreement with the K_M for serum HuPON1 (23 mM) (2).

The substrate specificity of PON1 is unusually broad; its catalytic efficiencies span five orders of magnitude between the best substrate known (phenylacetate) and L-HcyT (k_{cat}/K_M of 10^6 to 10 s⁻¹ M⁻¹). To establish that the various catalytic activities all occur at the same active site, we used the compet-

Table 1. Kinetic parameters and *E. coli* expression levels of the newly evolved rePON1 variants

Variant*	Phenylacetate hydrolysis			Paraoxon hydrolysis			Yield, purified enzyme mg/liter culture
	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹	
G1A5 [†]	833	0.39	2.1×10^6	1.16	0.085	1.4×10^4	2.2
G1C4 [†]	552	0.54	1.0×10^6	0.54	0.12	0.5×10^4	2.7
G2D6 [†]	562	0.32	1.7×10^6	0.98	0.10	1.4×10^4	14.4
G2E6 [†]	965	0.43	2.2×10^6	0.87	0.089	1.0×10^4	20
G3H8 [‡]	1018	0.32	3.2×10^6	1.2	0.088	1.4×10^4	11.8
G3C9 [‡]	789	0.33	2.4×10^6	1.1	0.094	1.2×10^4	12.6
Wild-type HuPON1 [§]	1236	0.42	2.9×10^6	3	0.54	0.6×10^4	0

*G1, G2, and G3 designate clones isolated after the first, second, and third round of evolution, respectively.

[†]These trx-rePON1 variants were expressed fused to thioredoxin by an S- and a 6×His tag.

[‡]These rePON1 variants contain the unmodified 355-aa PON1 protein devoid of any additions.

[§]Kinetic parameters for wild-type, serum-purified HuPON1 R form are taken from ref. 31. Wild-type RabPON1 is more suitable for comparison with the newly evolved PON1 variants. However, our attempts to purify wild-type RabPON1 from *E. coli* lysates gave an enzyme with >10-fold lower k_{cat} values (92 and 0.19 s⁻¹ for phenyl acetate and paraoxon, respectively) than HuPON1. Kinetic parameters for RabPON1 purified from sera have not been published, but Draganov *et al.* (13) and Kou and La Du (26) reported their close similarity to those of HuPON1. The reduced rate observed for RabPON1 expressed in *E. coli* may be due to its tendency to aggregate after purification, suggesting the presence of misfolded inactive protein in the purified samples. In contrast, the newly evolved variants were stable and did not aggregate or lose activity during months of storage at 4°C.

Table 2. Kinetic parameters of the newly evolved rePON3 variants

Variant*	Paraoxon hydrolysis			2NA hydrolysis		
	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹
RabPON3	0.001	1.3	0.73 (1)	0.66	0.211	3.1×10^3 (1)
MoPON3	0.0008	1.4	0.58	2.12	0.5	4.2×10^3
G1A7	0.007	2.5	2.9 (4)	1.9	0.16	1.2×10^4 (3.7)
G1B11	0.009	1.1	7.6 (10.4)	2	0.18	1.1×10^4 (3.5)
G2C2	0.036	0.8	45 (62)	18.8	0.26	7.2×10^4 (23)
G3A5	0.04	0.51	78.3 (107)	ND	ND	3.5×10^4 (11)
G3G3	0.11	0.75	156.6 (215)	26.25	0.65	4.0×10^4 (13)
G3H9	0.14	0.8	175 (240)	ND	ND	3.5×10^4 (11)

*All variants described above are trx-rePON3 variants, expressed fused to thioredoxin by 5- and 6×His tags. ND, not determined.

†Noted in parentheses is the fold improvement relative to trx-RabPON3.

itive inhibitor 2-hydroxyquinoline (11). Despite the vast differences in k_{cat} and K_M values, the inhibition constants for phenylacetate, paraoxon, and L-HcyT hydrolysis were found to be 3–11 μ M (Table 7 and Fig. 11, which are published as supporting information on the PNAS web site). These values agree with the inhibition constant of $\approx 5 \mu$ M measured for hydrolysis of phenylacetate by serum HuPON1 (11). The close similarity of the inhibition constants above is strong evidence that all three substrates are hydrolyzed at the same active site; however, the complete proof can come only from deciphering the enzyme structure and catalytic mechanism.

Newly Evolved PON3 Variants (rePON3). Relative to PON1, little is known about PON3. Because of its low level in the serum (≈ 50 -fold lower than PON1), PON3 was only recently purified and characterized (13, 14), and it was not expressed in heterologous expression systems. The specific kinetic parameters of serum PON3 have not been determined, but relative activities of various substrates of RabPON3 are available (13).

Unlike wild-type PON1, we found that wild-type genes of both rabbit and mouse PON3, when fused to thioredoxin, could be expressed in *E. coli* at reasonable levels (4–6 mg/liter) and appear to be stable and active. The serum-purified and *E. coli*-expressed PON3s show the same pattern of specificity with dihydrocoumarin (a lactone substrate) and the ester substrates phenyl and naphthyl acetate (Table 8, which is published as supporting information on the PNAS web site). However, we have observed very weak paraoxonase activity with both mouse and rabbit PON3. Activity was very low ($<0.1\%$ of that of PON1) and exhibited a prolonged lag (≈ 30 min) in onset (hysteresis). These two factors may account for the earlier reports of no paraoxonase activity in the serum-purified RabPON3 (13, 14). Thus, the enzymatic properties of RabPON3 are not significantly altered on fusion to thioredoxin and expression in *E. coli*, and the detailed kinetic parameters obtained here are, in general, relevant to wild-type PON3s.

Wild-type PON3s exhibit 100- to 1,000-fold lower rates of ester and phosphotriester hydrolysis than PON1, although lactonase activities of the two enzymes are similar (13, 14). Their sequences are $\approx 65\%$ identical at the amino acid level. We were interested to see how far the two enzymes had diverged and whether, under directed selection pressure, they might reconverge to exhibit similar, if not identical, phenotypes. Indeed, three rounds of shuffling and screening of wild-type PON3 genes yielded clones with greatly increased rates of both ester and phosphotriester hydrolysis. Comparison of selected variants from the three rounds of evolution (Table 2) indicated that the evolutionary process was directed primarily toward an increase

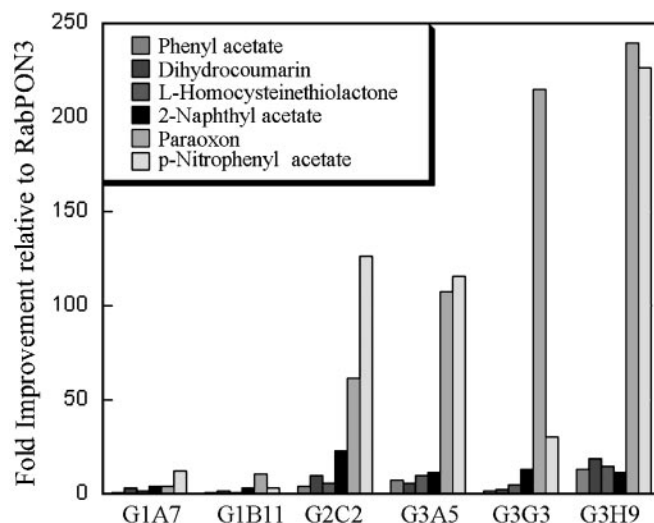


Fig. 2. Improvements in catalytic specificity (k_{cat}/K_M) toward various substrates of variants of trx-rePON3 from the different rounds of evolution (with respect to wild-type trx-RabPON3). G1A7 and G1B11 are first-generation variants, G2C2 is a second-generation variant, and G3A5, G3G3, and G3H9 are third-generation variants. The kinetic parameters are presented in Tables 2 and 9.

in the catalytic efficiency of PON3 (see also Table 9, which is published as supporting information on the PNAS web site). After the first round, a mild improvement in catalytic efficiency is observed (Table 2, variants G1A7 and G1B11). After the second round of evolution, one clone was isolated (G2C2) that exhibits (relative to wild-type RabPON3) 62- and 23-fold higher activity for paraoxon and 2NA hydrolysis, respectively. After the third round of evolution, three clones were isolated (G3A5, G3G3, and G3H9) that exhibit an overall improvement of up to 240-fold in paraoxonase activity. Thus, although wild-type PON3s exhibit almost no paraoxonase activity, shuffled variants of the same genes exhibit paraoxonase activity only 60-fold lower than PON1. However, no further improvement in 2NA activity was observed with these clones (Fig. 2 and Table 2). A 4- to 7-fold increase in the expression level of PON3s was also observed after three rounds of evolution (Table 9). Like PON1, the newly evolved PON3 could be expressed and purified (by adding a C-terminal 6×His tag; rePON3 variant G3H9) without the thioredoxin fusion protein, and its catalytic parameters were similar to those of trx-rePON3.

Kinetic parameters for other ester and lactone substrates (Table 9) revealed that the improvements in the catalytic efficiency can be divided into two groups. For the first group, which includes 2NA, phenylacetate, dihydrocoumarin, and L-HcyT, the increase in k_{cat}/K_M values for the third-generation variants is 2- to 20-fold (relative to the wild-type trx-reRabPON3). The second group includes paraoxon and *p*-nitrophenylacetate, for which the third-generation variants exhibit k_{cat}/K_M values up to 240-fold higher than wild-type PON3 (Fig. 2). Thus, the activity toward *p*-nitrophenylacetate co-evolved with that of paraoxon, whereas all the other substrates show a much milder improvement. In our view, this does not indicate the existence of two catalytic sites or subsites. Rather, the common feature of *p*-nitrophenylacetate and paraoxon is the *p*-nitrophenol-leaving group. The coevolution of the two substrates suggests that hydrolysis of both carboxy- and phosphoesters occurs by means of the same mechanism and at the same site.

Sequence analysis of the selected rePON3 variants indicates that the parental genes are evenly represented in variants of the

first round of evolution (Fig. 7B). Alignment of variants from the second and third rounds of evolution reveals that, although the sequence similarity between the selected clones is very low, the RabPON3 gene is mildly overrepresented relative to the HuPON3 and MoPON3 genes. These subtle patterns of convergence in the sequences of the selected PON3 are in contrast to the clear convergence of the PON1 newly evolved variants. They show that many routes exist by which the enzymatic activity of PON3 can be increased.

Newly Evolved OP-Selective rePON1 Variants. Gene libraries of the highly soluble rePON1 variant G3C9 were prepared by random mutagenesis by using the wobble-base PCR method with either dPTP or 8-oxo-dGTP nucleoside analogs (22). The number of mutations per gene was adjusted by varying the concentration of dNTP analogs and the number of PCR cycles. Five libraries were generated: two dPTP libraries with 98% transitions, an average of 5–14 mutations per gene, and 20% to 6% residual activity in the pools of genes, respectively; and three 8-oxo-dGTP libraries with an average of 85% transversions, 3–10 mutations per gene, and 13% to 7% residual activity. In addition to the plate screen for 2NA hydrolysis described above, the libraries were directly screened on agar plates (with no need for replication) for the hydrolysis of a newly synthesized fluorogenic OP substrate, DEPCyC (Fig. 4). This substrate, which is a close homologue of the insecticide coumaphos previously used for the directed evolution of a bacterial phosphotriesterase (28, 29), exhibits higher fluorescence at a more convenient wavelength. Screening of $\approx 10^3$ to 10^4 colonies from each library with 2NA yielded variants with mildly improved activity against OP substrates. The best variants were reshuffled and screened directly with DEPCyC, yielding a highly improved variant, reG3C9.49, that carried four mutations: G19R, S193P, N287D, and V346A. The V346A mutation appeared in other improved variants, suggesting its primary role in the enhancement of DEPCyC-hydrolyzing activity. The first-generation 8-oxo-dGTP libraries were also screened directly with DEPCyC to yield another highly improved variant, reG3C9.10, which contained two mutations, L69V and E218D. The two clones were purified and analyzed in detail (Fig. 3 and Table 3). The mutations affected the specific activity toward DEPCyC and phenylacetate, whereas the expression level of the two selected variants was not altered. Both variants exhibited a ≈ 40 -fold increase in catalytic proficiency (k_{cat}/K_M) toward DEPCyC compared with wild-type PON1 (and its recombinant variant G3C9), and a simultaneous 50-fold reduction in catalytic proficiency toward phenylacetate. The change in the catalytic efficiencies resulted primarily from changes in k_{cat} values for both substrates. Both variants showed a similarly reduced activity with other esters (e.g., 2NA) and mildly improved activity with another OP substrate (paraoxon; data not shown).

Conclusions and Implications

The outcome of the *in vitro* evolutionary process described above is a range of recombinant PON variants with a broad range of enzymatic properties. Expression of these variants in *E. coli* clearly demonstrates that, in contrast to previous findings (16), glycosylation is not an absolute requirement for the stability or enzymatic functions of PON1 and PON3. Expression in *E. coli* provides an ample source of PON1 and PON3 free from other serum proteins and will facilitate mechanistic and structural characterization of these important enzymes. We were already able to establish unequivocally that homocysteine lactonase activity, which is probably the weakest activity ascribed to the serum-purified enzyme (2, 11) ($\approx 10^5$ -fold weaker than its activity toward phenylacetate), takes place at the same catalytic site at which paraoxon and phenylacetate hydrolysis occur.

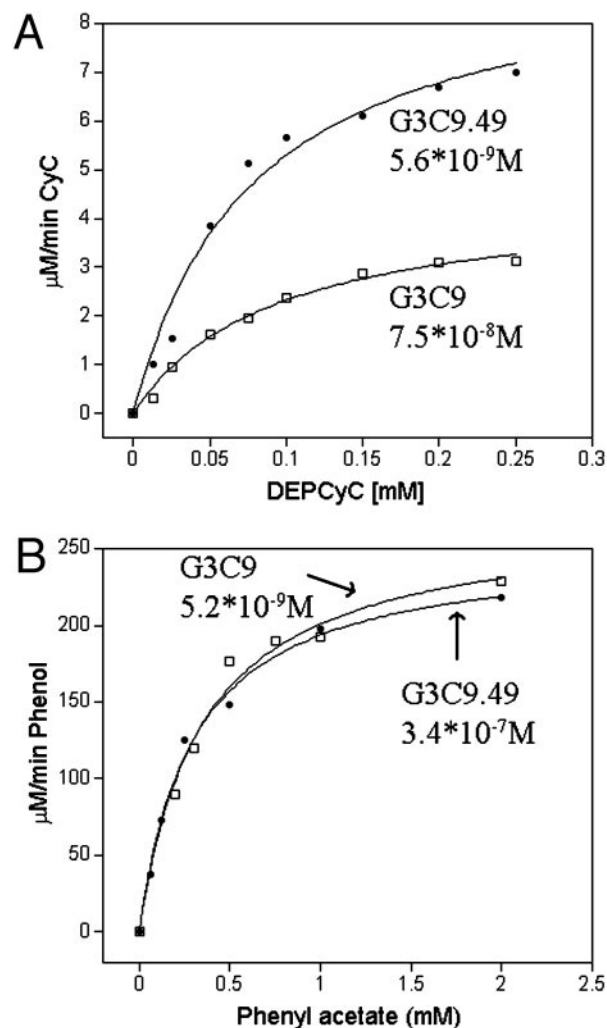


Fig. 3. Michaelis-Menten plots for the hydrolysis of DEPCyC (A) and phenylacetate (B) by rePON1 variant G3C9 and its variant G3C9.49 that displays greatly enhanced OP-hydrolyzing activity. Enzyme concentrations are 7.5×10^{-8} M and 5.6×10^{-9} M in A and 5.2×10^{-9} M and 3.4×10^{-7} M in B for G3C9 and G3C9.49, respectively. The kinetic parameters derived from the fits are presented in Table 3.

PON1 and PON3 (as well as PON2) are members of the same gene family, but what caused an ancestral PON gene to duplicate and diverge in the course of natural evolution is not known (30). It is also unclear whether PON3 specializes in an activity that PON1 lacks or vice versa. Here we show that their responses to *in vitro* selection pressure toward higher paraoxonase and esterase activity are quite different. PON1 evolved to higher solubility, whereas its enzymatic properties seemed to have remained the same. In contrast, PON3 evolved primarily to higher catalytic efficiency; wild-type PON3s have almost no paraoxonase activity, but shuffled variants of the same genes exhibit paraoxonase activity only 60-fold lower than that of PON1. The sequence variations observed for PON1 and PON3 during the directed evolutionary process are also markedly different: whereas the newly evolved PON3 genes show considerable divergence, PON1 rapidly converged toward the rabbit sequence that seems to encode the most soluble and stable PON1. This example shows how closely related genes can behave very differently under the same selection pressure. The outcome of this directed selection pressure also indicates that, despite their divergence in natural evolution, PON1 and PON3 remain very close, not only in

Table 3. Kinetic parameters for variants of rePON1 selected from random mutagenesis libraries for OP hydrolysis

Variant*	DEPCyC hydrolysis [†]			Phenylacetate hydrolysis			Selectivity factor k_{cat}/K_M (DEPCyC)/ k_{cat}/K_M (PA)
	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹	k_{cat} , s ⁻¹	K_M , mM	k_{cat}/K_M , M ⁻¹ s ⁻¹	
G3C9	0.8	0.07	1.1×10^4	789	0.33	2.4×10^6	0.0046
G3C9.10	25.7	0.05	4.8×10^5	39.6	0.76	5.2×10^4	9.2
G3C9.49	28.1	0.08	3.6×10^5	12.4	0.3	4.1×10^4	8.8

*All rePON1 variants above comprise the unmodified 355-aa PON1 protein without any additions.

[†]See Fig. 4.

sequence but also in active-site structure and catalytic mechanism. This closeness is best exemplified by the rapid regain of paraoxonase activity in PON3 after the selection pressure.

Random mutagenesis and direct selection for OP hydrolysis led to >40-fold improvement in the catalytic proficiency of PON1 toward the phosphotriester DEPCyC (Table 3). This increase was accompanied by a >2,000-fold shift in specificity that was not selected for. Wild-type PON1 is essentially an esterase with very weak phosphotriesterase activity (k_{cat}/K_M for phenylacetate is 220- to 480-fold higher than for paraoxon or DEPCyC). The directly evolved variants were converted into a phosphotriesterase with weak esterase activity (k_{cat}/K_M for DEPCyC is 10-fold higher than for phenylacetate). Also notable is the exceptionally subtle nature of mutations that lead to the shift in specificity and the fact that mutations at two different

locations (V346A in reG3C9.49 vs. L69V and E218D in reG3C9.10) produce the same shift. Finally, the demonstration that PONs are amenable to the powerful tool of directed evolution opens new prospects for improving their phosphotriesterase activity toward other hazardous OPs and toward catalytic activities related to the prevention of atherosclerosis.

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- La Du, B. N., Aviram, M., Billecke, S., Navab, M., Primo-Parmo, S., Sorenson, R. C. & Standiford, J. J. (1999) *Chem. Biol. Interact.* **119–120**, 379–388.
- Jakubowski, H. (2000) *J. Biol. Chem.* **275**, 3957–3962.
- Davies, H. G., Richter, R. J., Keifer, M., Broomfield, C. A., Sowalla, J. & Furlong, C. E. (1996) *Nat. Genet.* **14**, 334–336.
- Biggadike, K., Angell, R. M., Burgess, C. M., Farrell, R. M., Hancock, A. P., Harker, A. J., Irving, W. R., Ioannou, C., Procopiou, P. A., Shaw, R. E., et al. (2000) *J. Med. Chem.* **43**, 19–21.
- Smolen, A., Eckerson, H., Gan, K., Hailat, N. & La Du, B. (1991) *Drug Metab. Dispos.* **19**, 107–112.
- Shih, D. M., Gu, L. J., Xia, Y. R., Navab, M., Li, W. F., Hama, S., Castellani, L. W., Furlong, C. E., Costa, L. G., Fogelman, A. M. & Lusic, A. J. (1998) *Nature* **394**, 284–287.
- Lusic, A. J. (2000) *Nature* **407**, 233–241.
- Ahmed, Z., Ravandi, A., Maguire, G. F., Emili, A., Draganov, D., La Du, B. N., Kuksis, A. & Connelly, P. W. (2001) *J. Biol. Chem.* **276**, 24473–24481.
- Aviram, M., Rosenblat, M., Bisgaier, C. L., Newton, R. S., Primo-Parmo, S. L. & La Du, B. N. (1998) *J. Clin. Invest.* **101**, 1581–1590.
- Rodrigo, L., Mackness, B., Durrington, P. N., Hernandez, A. & Mackness, M. I. (2001) *Biochem. J.* **354**, 1–7.
- Billecke, S., Draganov, D., Counsell, R., Stetson, P., Watson, C. & La Du, B. N. (2000) *Drug Metab. Dispos.* **28**, 1335–1342.
- Marathe, G., Zimmerman, G. & McIntyre, T. (2003) *J. Biol. Chem.* **278**, 3937–3947.
- Draganov, D. I., Stetson, P. L., Watson, C. E., Billecke, S. S. & La Du, B. N. (2000) *J. Biol. Chem.* **275**, 33435–33442.
- Reddy, S. T., Wadleigh, D. J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Shih, D. M., Lusic, A. J., Navab, M. & Fogelman, A. M. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21**, 542–547.
- Josse, D., Lockridge, O., Xie, W. H., Bartels, F., Schopfer, L. M. & Masson, P. (2001) *J. Appl. Toxicol.* **21**, S7–S11.
- Brushia, R. J., Forte, T. M., Oda, M. N., La Du, B. & Bielicki, J. K. (2001) *J. Lipid Res.* **42**, 951–958.
- Josse, D., Ebel, C., Stroebel, D., Fontaine, A., Borges, F., Echaliier, A., Baud, D., Renault, F., le Maire, M., Chabrieres, E. & Masson, P. (2002) *J. Biol. Chem.* **277**, 33386–33397.
- Waldo, G. S. (2003) *Curr. Opin. Chem. Biol.* **7**, 33–38.
- Farinas, E. T., Bulter, T. & Arnold, F. H. (2001) *Curr. Opin. Biotechnol.* **12**, 545–551.
- Tao, H. & Cornish, V. W. (2002) *Curr. Opin. Chem. Biol.* **6**, 858–864.
- Abecassis, V., Pompon, D. & Truan, G. (2000) *Nucleic Acids Res.* **28**, E88.
- Zaccolo, M., Williams, D. M., Brown, D. M. & Gherardi, E. (1996) *J. Mol. Biol.* **255**, 589–603.
- Khalamezyer, V., Fischer, I., Bornscheuer, U. & Altenbuchner, J. (1999) *Appl. Environ. Microbiol.* **65**, 477–482.
- Hammarstrom, M., Heggren, N., van Den Berg, S., Berglund, H. & Hard, T. (2002) *Protein Sci.* **11**, 313–321.
- Cramer, A., Raillard, S. A., Bermudez, E. & Stemmer, W. P. (1998) *Nature* **391**, 288–291.
- Kuo, C. L. & La Du, B. N. (1995) *Drug Metab. Dispos.* **23**, 935–944.
- Li, W. F., Costa, L. G., Richter, R. J., Hagen, T., Shih, D. M., Tward, A., Lusic, A. J. & Furlong, C. E. (2000) *Pharmacogenetics* **10**, 767–779.
- Harcourt, R. L., Horne, I., Sutherland, T. D., Hammock, B. D., Russell, R. J. & Oakeshott, J. G. (2002) *Let. Appl. Microbiol.* **34**, 263–268.
- Yang, H., Carr, P. D., McLoughlin, S. Y., Liu, J. W., Horne, I., Qiu, X., Jeffries, C. M., Russell, R. J., Oakeshott, J. G. & Ollis, D. L. (2003) *Protein Eng.* **16**, 135–145.
- Primo-Parmo, S. L., Sorenson, R. C., Teiber, J. & La Du, B. N. (1996) *Genomics* **33**, 498–507.
- Josse, D., Xie, W. H., Renault, F., Rochu, D., Schopfer, L. M., Masson, P. & Lockridge, O. (1999) *Biochemistry* **38**, 2816–2825.