Structure−Reactivity Studies of Serum Paraoxonase PON1 Suggest that Its Native Activity Is Lactonase†

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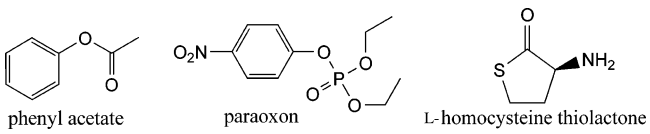
ABSTRACT: PON1 is the best-studied member of a family of enzymes called serum paraoxonases, or PONs, identified in mammals (including humans) and other vertebrates as well as in invertebrates. PONs exhibit a range of important activities, including drug metabolism and detoxification of organophosphates such as nerve agents. PON1 resides on HDL (the "good cholesterol") and is also involved in the prevention of atherosclerosis. Despite this wealth of activities, the identity of PON1's native substrate, namely, the substrate for which this enzyme and other enzymes from the PON family evolved, remains unknown. To elucidate the substrate preference and other details of PON1 mechanism of catalysis, structure−activity studies were performed with three groups of substrates that are known to be hydrolyzed by PON1: phosphotriesters, esters, and lactones. We found that the hydrolysis of aryl esters is governed primarily by steric factors and not the pK_a of the leaving group. The rates of hydrolysis of aliphatic esters are much slower and show a similar dependence on the pK_a of the leaving group to that of the nonenzymatic reactions in solution, while the aryl phosphotriesters show much higher dependence than the respective nonenzymatic reaction. PON1-catalyzed lactone hydrolysis shows almost no dependence on the pK_a of the leaving group, and unlike all other substrates, lactones seem to differ in their K_M rather than k_{cat} values. These, and the relatively high rates measured with several lactone substrates ($k_{cat}/K_M \approx 10^6$ M^{-1} s^{-1}) imply that PON1 is in fact a lactonase.

Serum paraoxonase (PON1) is a mammalian enzyme with hydrolase activity toward multiple substrates (*1*, *2*). PON1 is a member of a family of enzymes that are widely spread in mammals, such as rat, rabbit, and mouse, as well as humans, but are also found in many other species including *Caenorhabditis elegans* (*1*). The mammalian PONs[1] are divided into three subfamilies that share 60−70% amino acid identity. PON1 and PON3 are mainly expressed in the liver and reside on the cholesterol-carrying particles HDL (the "good cholesterol"), while PON2 is expressed in many tissues (*3*). PON1 is by far the most investigated member of the family and became the subject of intensive research owing to its ability to inactivate various organophosphates, including nerve gases and pesticides, which present both an environmental risk and a terrorist threat. The name is derived from paraoxon (Chart 1), the metabolite of the common pesticide parathion, which is hydrolyzed by PON1 with modest catalytic efficiency ($k_{cat}/K_M < 10^4$ M^{-1} s^{-1}). PON1 has also been shown to be involved in drug metabolism and is used for drug inactivation (*4*). Research in the past decade has shown that PON1 has antiatherosclerotic activity (*5*). In vitro assays indicated that it inhibits lipid oxidation of the low-density lipoprotein (LDL) and mediates the efflux of cholesterol from macrophages. But these activities have not been directly linked with PON1's hydrolytic activities (*5*−*8*). A possible physiological substrate is homocysteine thiolactone, which is a known risk factor in the atherosclerotic vascular diseases. The only serum homocysteine thiolactonase activity identified thus far is that of PON1 (*9*), although this activity is rather low ($k_{cat}/K_M \approx 10$ M^{-1} s^{-1}) (*10*). PON1 has an appreciable aryl esterase activity, with phenyl acetate being a typical substrate ($k_{cat}/K_M \approx 10^6$ M^{-1} s^{-1}). The antiatherosclerotic activity has also been associated with phospholipase A2 (PLA2)-like activity (*11*), but this observation has later been ascribed to contaminations rather than genuine PON1 activity (*12*, *13*).

Chart 1



Structural and functional characterization of the PONs and their engineering, were hindered by lack of an ample source of recombinant protein. We recently described the directed evolution of several recombinant PON1 and PON3 variants that express in a soluble and active form in *Escherichia coli* and exhibit enzymatic properties almost identical to those reported for PONs purified from sera (*10*). A crystal structure of one of these variants (rePON1 G2E6) was solved, providing the first structure of a PON family member (*14*).

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[1] Abbreviations: PON, serum paraoxonase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PLA2, phospholipase A2; PTPase, protein-tyrosine phosphatase; PTE, bacterial phosphotriesterase; LFER, linear free energy relationships; SAR, structure−activity relationships.

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PON1 was found to be a six-bladed β-propeller with two Ca^{2+} ions in its central tunnel. One calcium atom lies at the bottom of the active site and is postulated to play a role in catalysis, while the inner calcium is largely buried and appears to have a structural function. On the basis of pH-rate profiles, an unprotonated histidine was proposed to play an important role in catalysis. The structure indicated a general-base mechanism reminiscent of secreted PLA2 (*15*): an activation of a water molecule by a histidine side chain, followed by a nucleophilic attack at the phosphoryl/carbonyl center of the substrates. The negative charge of the resulting intermediates (and the respective transition states) is probably stabilized by the catalytic calcium. However, the absence of detailed mechanistic studies and structural data regarding the enzyme−substrate complexes prevented a more detailed description of PON1's mechanism.

Previous structure−activity relationships (SAR) of PON1 (*16*) did not provide detailed insight regarding its catalytic mechanism. PON1 hydrolyzes a wide range of substrates, such as esters, thioesters, phosphotriesters, carbonates, lactones, and thiolactones. The highest activities observed thus far are with synthetic substrates such as phenyl acetate and dihydrocoumarin ($k_{cat}/K_M \geq 10^6$ M^{-1} s^{-1}) (*1*, *10*) that have no physiological relevance. It is therefore unlikely that these are PON1's native substrates. Recently, lactonase (lactone hydrolysis) as well as lactonizing (lactone formation) activities of PON1 were described, including those with lactones of potential physiological relevance such as products of fatty acid oxidation (*17*, *18*). These results imply that PON1 might in fact be a lactonase rather than an aryl-esterase or paraoxonase, as traditionally described.

We probed the steric and the electronic requirements of the active site of PON1, with the aim of identifying its native activity; that is, the activity for which PON1 evolved and for which its active site is tailored. Structure−activity relationships and linear free energy relationships (LFERs), in particular, constitute an important tool in probing the mechanisms of enzymatic and nonenzymatic reactions. They can provide information about the rate-determining steps of the reaction, as well as the nature of the transition states involved. Despite the complexity of LFER studies of enzymes and the difficulty of separating steric effects on substrate binding from effects on catalysis (transition state stabilization), these studies provide meaningful insights for many enzymes, including β-glycosidases (*19*), alkaline phosphatase (*20*), PTPase (*21*), bacterial phosphotriesterase (PTE) (*22*), and phospholipase C (*23*). We applied the Brønsted LFER to PON1 and varied the pK_a of the leaving groups of the substrates. We examined more than 50 different substrates belonging to three different classes: esters, phosphotriesters, and lactones. Our results suggest that PON1 is not an esterase nor a phosphotriesterase but rather a lactonase.

MATERIALS AND METHODS

General. Chemicals were purchased from Aldrich Chemicals Company, Fluka, and Acros Chemicals. Kinetics were performed with recombinant PON1 variant rePON1−G2E6 expressed in fusion with a thioredoxin and 6His tag and purified as described (*10*).

Synthesis. 3,5-Dinitrophenol was prepared from 3,5-dinitroaniline by the method of Cohen et al. (*24*). 1-Phenylvinyl acetate was prepared from acetophenone (*25*). The diethylphosphoryl derivatives **1**−**15** were synthesized from diethyl phosphochloridate and the corresponding phenols. The bases in the reaction were chosen according to the pK_a of the phenol (triethylamine (compounds **5**, **7**−**15**), DMAP (compound **4**), pyridine (compound **1**), and *N*-methyl morpholine (compounds **2** and **3**)). Substituted phenyl acetate derivatives **16**−**28**, **30**, **31**, and **33** were prepared by reacting the corresponding phenols with acetyl chloride using triethylamine as a base. Aliphatic esters **34**, **35**, and **36** were synthesized from the corresponding alcohols and acetyl chloride without a base. The structure and purity of all the synthesized compounds were verified by ^{1}H NMR spectroscopy. The detailed procedures and spectra can be found in the Supporting Information. The pK_a values of aliphatic alcohols (*23*, *26*), benzylic alcohols (*27*, *28*), and phenols (*22*, *23*, *29*−*31*) were obtained from the literature. The pK_a value of 3,5-dinitrophenol was determined spectrophotometrically. The λ_{max} and extinction coefficients of the substituted phenols and the corresponding diethyl phosphoryl and acetyl derivatives were determined spectrophotometrically at pH 8.0 on a microtiter plate reader (PowerWave HT microplate scanning spectrophotometer; optical length ~0.5 cm) and are presented in the Tables 1 and 2.

Kinetic Measurements. The rates of enzymatic hydrolyses of the phosphotriester substrates **1**−**15**, of phenolic esters **16**−**31**, **33**, and of dihydrocoumarin (**51**) were determined at pH 8.0−8.3 where the pH-rate profile of PON1 is at plateau (*14*), in 0.1 M bis-trispropane with 1 mM $CaCl_2$. The ionic strength was adjusted to a total of 0.2 M with NaCl. The enzyme stocks were kept in Tris 50mM, containing 0.1% tergitol, 50 mM NaCl, and 1 mM $CaCl_2$. A range of enzyme concentrations were used ($[E]_o = 1.25 \times 10^{-8}$−$2.5 \times 10^{-6}$ M) depending on the reactivity of the substrate. Stocks of 500 mM of substrates were prepared in MeOH (a 100 mM dihydrocoumarin stock in DMSO was used) and diluted with the reaction buffer immediately before initializing the reaction. The substrate concentrations were varied in the range of $0.3 \times K_M$ up to $(2-3) \times K_M$, except for the cases where substrate solubility was limiting (compounds **3**, **20**, **24**, **32**, **34**, **36**, and **40**). The cosolvent percentage was kept at 1% or 1.6%, in all reactions. Cosolvent percentage had to be kept constant, since various organic solvents were found to inhibit PON1's activity. DMF exhibited the most severe inhibition, while methanol had the mildest influence on the kinetic parameters. The participation of solvents as reactants (e.g., methanolysis) was ruled out by comparing the rates of phenyl acetate hydrolysis by rePON1 with 1, 2, and 5% of various cosolvents, both by absorbance of the released phenol leaving group and by the pH-indicator assay. For further information, see Tables 1−3 in Supporting Information. The K_M values observed in kinetic runs, in which the percentage of cosolvent increased with substrate concentration, were several times lower than in runs in which the cosolvent concentration was kept the same in all substrate concentrations. Variations in k_{cat} values were less pronounced, and when the cosolvent percentage was equalized, the k_{cat} values increased up to 2-fold. Consequently, there were little variations, if any, of the k_{cat}/K_M values, and the general picture of the reactivity plots remained the same. This sensitivity to cosolvent also accounts for the differences in the kinetic parameters initially reported for rePON1−G2E6

(*10*, *14*) and for the serum-purified human PON1 (*32*) and those reported here (see footnotes to Tables 1 and 2). Product formation was monitored spectrophotometrically in 200 μL reaction volumes, using 96-well plates (polystyrene, at ≥320 nm, and quartz < 320 nm). Initial velocities (v_0) were determined at eight different concentrations for each substrate. For substrates **1** and **16**−**25**, v_0 values were corrected for the background rate of spontaneous hydrolysis in the absence of enzyme.

The pH Indicator Assay. The hydrolysis of lactones **42**−**50**, benzyl acetate (**37**), 1-phenylvinyl acetate (**32**), and the aliphatic esters (**34**−**36** and **38**−**41**) was monitored by a pH-sensitive colorimetric assay (*33*). Proton release from carboxylic acid formation was followed using the pH indicator cresol purple. The reactions were performed at pH 8.0−8.3 in bicine buffer 2.5 mM, containing 1 mM $CaCl_2$ and 0.2 M NaCl. The reaction mixture contained 0.2−0.3 mM cresol purple (from a 60 mM stock in DMSO). Upon mixture of the substrate with the enzyme, the decrease in absorbance at 577 nm was monitored in a microtiter plate reader. The assay required in situ calibration with acetic acid (standard acid titration curve), which gave the rate factor (−OD/mole of H^+). The enzyme stocks were kept in Tris 50 mM, containing 0.1% tergitol, 50 mM NaCl, and 1 mM $CaCl_2$. The presence of detergent caused a decrease in enzymatic rates. However, prolonged storage of enzyme stock in buffer with low tergitol concentration (0.01%) induced a change in kinetic parameters. Thus, enzyme stocks were diluted 50-fold into bicine buffer immediately before the kinetic measurements. In the case of aliphatic esters, the slow rates required higher enzyme concentrations, and calibration of the assay was performed with addition of the buffer from the enzyme stock, yielding lower rate factors. Lactone substrates **42**−**50** were added from stocks of 500 mM in DMSO, and the percentage of DMSO was kept constant at 1% in all reactions regardless of the initial substrate concentration. DMSO was chosen as a cosolvent for lactone stock solutions, since γ-undecanoic lactone (**47**) and γ-dodecanoic lactone (**48**) were not soluble in methanol. Both γ-undecanoic lactone (**47**) and γ-dodecanoic lactone (**48**) were dissolved in buffer with Triton X-100 detergent at a final concentration of 0.007−0.06%. The presence of Triton caused a decrease in rates, and the system had to be calibrated for the addition of Triton (the rate factor was determined in the presence of 0.05% Triton). A stock solution of trifluoroethyl acetate (**34**) was also prepared in DMSO, but other aliphatic esters could be dissolved without cosolvent. The differences in the kinetic parameters with and without cosolvent in this case were not larger than 10%. A background rate was observed in the absence of any substrate presumably due to acidification by atmospheric CO_2. This rate (4−11 mOD/min), that was independent of substrate concentration, was subtracted from all v_0 values.

Inhibition. The inhibition by 2-hydroxyquinoline, of paraoxon (**6**), phenyl acetate (**29**), and δ-valerolactone (**49**) hydrolysis by rePON1 was determined by building double reciprocal plots (Lineweaver−Burk) at two inhibitor concentrations and without an inhibitor. Substrate concentrations were 0.5−4.0 mM, with 1% cosolvent (methanol, in the case of paraoxon, and phenyl acetate and DMSO, in the case of δ-valerolactone); enzyme concentrations were 8.37×10^{-9} M with phenyl acetate, 1.675×10^{-7} M with paraoxon, and 8.375×10^{-9}−1.675×10^{-8} M with δ-valerolactone.

Viscosity Experiments. Kinetic assays were performed with glycerol (0−27%, w/w) and sucrose (0−30%, w/w) added to 50mM Tris at pH 8.15, containing 1 mM $CaCl_2$ and 50 mM NaCl. The relative viscosity (η/η_0) of the solutions was obtained from literature (*29*, *34*).

Data Analysis. Kinetic parameters (k_{cat}, K_M, k_{cat}/K_M, K_i) were obtained by fitting the data to the Michaelis−Menten equation [$v_0 = k_{cat}[E]_0[S]_0/([S]_0 + K_M)$] and to the competitive inhibition model [$v_0 = v_{max}[S]_0/([S]_0 + K_M(1 + ([I]/K_i))$] (using the reciprocal form $\{1/v_0 = 1/[V_{max} + (K_M/V_{max}[S])-(1 + [I]/K_I)]\}$). Fitting was performed with the program Kaleidagraph 5.0. In cases where the solubility limited the substrate concentrations (compounds **3**, **20**, **24**, **32**, **34, 36**, and **40**), data were fitted to the linear regime of the Michaelis−Menten model [$v_0 = [S]_0[E]_0k_{cat}/K_M$], and k_{cat}/K_M was deduced from the slope. When possible, k_{cat} and K_M values for these compounds were also deduced from the fit to the Michaelis−Menten equation and are presented in Tables 1 and 2. All the data presented are the averages of at least three independent experiments, and standard deviations were calculated using Microsoft Excell 2003.

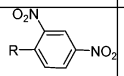
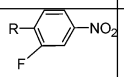
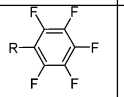
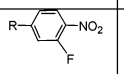
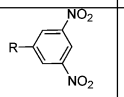
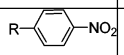
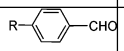
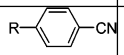
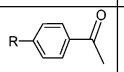
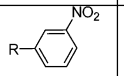
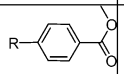
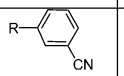
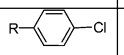
Hydrophobicity coefficients (log *P* values) of various ester substrates were determined by the ChemDraw Ultra 7.0 program.

RESULTS

The Phosphotriesterase Activity of PON1. The values of k_{cat} and K_M were determined for 15 analogues of *p*-nitrophenyl diethyl phosphate (**6**) (paraoxon, the substrate from which PON1 takes its name) and are listed in Table 1. These substrates differ only in the structure and the pK_a of the leaving group phenol. A Brønsted plot of log k_{cat} versus the leaving group pK_a (Figure 1A) shows extensive scatter. We excluded a number of substrates for steric reasons (compounds **1**−**3** and **7**; see below). The remaining substrates reveal that substrates with leaving group pK_a values of 7.14−9.38 exhibit a roughly linear relationship between log k_{cat} and the leaving group pK_a, with a negative slope of ca. −1.6. For substrates with pK_a values below 7.14, the dependence of the activity on the pK_a of the leaving group is much less pronounced, and the rates tend to plateau at pK_a < 6.6. A very similar picture was observed when the log(k_{cat}/K_M) values were plotted versus the leaving group pK_a (Figure 1B). In this case, the linear relationship observed for substrates with leaving group pK_a values of 7.14−9.38 corresponds to a slightly more negative β value of −1.76, and a clear plateau is observed at pK_a < 7.14. The similarity in β_{LG} values for the log(k_{cat}) and log(k_{cat}/K_M) plots (Figure 1, parts A and B, respectively) is in agreement with the K_M values of all phosphotriester substrates being in the range of 1.5 ± 0.5 mM, with minor exceptions (substrate (**6**), K_M = 0.8 mM and (**3**), K_M = 4.3 mM). Notably, the β_{LG} value of −1.6 is much lower than the value observed for the nonenzymatic (hydroxide-catalyzed) hydrolysis of phosphotriesters ($\beta_{LG} = -0.44$ (*29*)). The Brønsted plots also seem to plateau at rather low values: $k_{cat} \approx 10\ s^{-1}$ and $k_{cat}/K_M \approx 0.8 \times 10^4\ M^{-1}\ s^{-1}$. Both observations are inconsistent with PON1's native activity being of a phosphotriesterase.

Several substrates deviate from the above trend, apparently due to substituents on the ortho position of the phenol ring.

Table 1: Kinetic Parameters for Phosphotriesters Hydrolysis by rePON1 G2E6

Compound	**Formula** **R=** diethyl phosphate 	**pKa**	**λmax** **nm**	**Є** **OD/M**[a]	**k_{cat}** **s^{-1}**	**K_M** **mM**	**k_{cat}/K_M** **s^{-1} M^{-1}**
2,4 dinitrophenyl diethyl phosphate [**1**]		4.08[b]	360	9,160	0.27± 0.04	2.1 ± 0.5	130 ± 25
2-fluoro 4-nitrophenyl diethyl phosphate [**2**]		5.45[c]	395	11,080	3.7 ± 0.3	0.91 ± 0.08	4,040 ± 120
Pentafluorophenyl diethyl phosphate [**3**]		5.53[d]	270	678	~ 0.6	~ 4	~ 150 Linear fit[e]: 122 ± 15
3-fluoro 4-nitrophenyl diethyl phosphate [**4**]		5.94[c]	390	11,676	13.9 ± 0.4	1.7 ± 0.2	8,300 ± 600
3,5 dinitrophenyl diethyl phosphate [**5**]		6.59[f]	400	1,477	10.2 ± 0.2	1.8 ± 0.2	5,600 ± 660
4-nitrophenyl diethyl phosphate [**6**][g]		7.14[d]	405	10,515	4.8 ± 0.3	0.8 ± 0. 1	5,800 ± 370
2,6 difluorophenyl diethyl phosphate [**7**]		7.30[d]	270	950	0.062 ± 0.006	1.3 ± 0.3	48 ± 8
4-diethyl phosphate benzaldehyde [**8**]		7.66[d]	330	12,620	0.47 ± 0.03	1.20 ± 0.08	400 ± 21
4-cyanophenyl diethyl phosphate [**9**]		7.95[d]	275	7,495	0.40 ± 0.04	1.9 ± 0.4	220 ± 24
4-diethyl phosphate acetophenone [**10**]		8.05[d]	320	8,080	0.44 ± 0.02	1.56 ± 0.07	285 ± 8
3-nitrophenyl diethyl phosphate [**11**]		8.39[d]	340	840	0.076 ± 0.002	1.3 ± 0. 2	58 ± 8
4-diethyl phosphate methyl benzoate [**12**]		8.47[d]	295	5,485	0.244 ± 0.009	1.9 ± 0.4	130 ± 30
3-cyanophenyl diethyl phosphate [**13**]		8.61[d]	295	1,610	0.178 ± 0.003	2.1 ± 0.3	88 ± 11
3-fluorophenyl diethyl phosphate [**14**]		9.28[d]	270	656	1.112×10^{-3}± 4×10^{-6}	2.0 ± 0.1	0.57 ± 0.04
4-chlorophenyl diethyl phosphate [**15**]		9.38[d]	280	848	7.72×10^{-4}± 5×10^{-5}	1.9 ± 0.1	0.403 ± 0.003

[a] The λ_{max} and extinction coefficients of the respective substituted phenols products were determined spectrophotometrically at pH 8.0 on a microtiter plate reader (PowerWave HT microplate scanning spectrophotometer; optical length, ~0.5 cm). [b] From ref *30*. [c] From ref *29*. [d] From ref *22*. [e] A reliable fit to the Michaelis−Menten equation was not possible due to limited substrate solubility (maximal substrate concentration was $<2 \times K_M$). The approximate values are the values obtained from fit to the Michaelis−Menten equation [$v_0 = k_{cat}[E]_0[S]_0/([S]_0 + K_M)$], and the accurate k_{cat}/K_M values were obtained from a linear fit in the pseudo-second-order region of the Michaelis−Menten model [$v_0 = [S]_0[E]_0k_{cat}/K_M$]. [f] The p$K_a$ was determined spectrophotometrically by measuring the absorbance at 400 nm at pH 5.0−8.0. [g] The kinetic parameters previously reported by our group ($k_{cat} = 0.87\ s^{-1}$, $K_M = 0.089$ mM, and $k_{cat}/K_M = 1.0 \times 10^4\ M^{-1}\ s^{-1}$, (*10*)) were obtained using paraoxon stocks in DMF without equalizing the solvent percentage in all the substrate concentrations (see Materials and Methods).

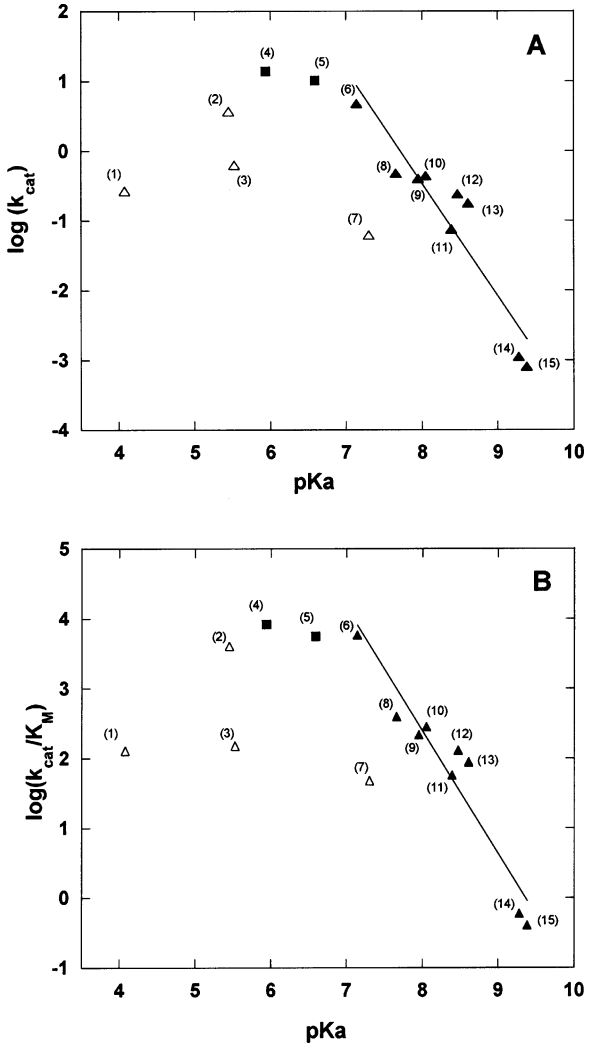


FIGURE 1: Structure−activity relationships for the hydrolysis of phosphotriesters by rePON1. Plotted are the logarithmic values of k_{cat} (A) and k_{cat}/K_M (B) vs pK_a of the leaving group. Substrates with ortho substituents on their phenol leaving groups give much lower rates than expected by their leaving group pK_a values and are shown as empty triangles. All other substrates are shown in full signs: those with leaving group pK_a > 7 (full triangles) were fitted to a line with slope equal to −1.62 (R = 0.94) for k_{cat} data and slope equal to −1.76 (R = 0.96) for k_{cat}/K_M data, while substrates with pK_a < 7 (full squares) exhibit a much milder slope with a β_{LG} value approaching zero. Numbers refer to the substrates entry in Table 1.

The k_{cat} values of 2,4-dinitrophenyl (**1**), 2-fluoro 4-nitrophenyl- (**2**), pentafluorophenyl- (**3**), and 2,6-difluorophenyl-diethyl phosphate (**7**) are much lower than expected from the pK_a of their leaving groups. The k_{cat} of the 2,6-difluorophenyl substrate (**7**), which is substituted at both ortho positions, is 77 times lower than that of paraoxon (**6**), despite their pK_a values being very similar (7.14 and 7.3, respectively).

The Esterase Activity of PON1. Phenyl acetate is the most commonly used ester substrate of PON1 ($k_{cat}/K_M \approx 10^6$ M^{-1} s^{-1}), and it is widely used to determine its activity. However, PON1's activity with other aryl esters has not been examined. Whether PON1 can hydrolyze aliphatic esters such as lipids is still questionable (*7*, *11*). We therefore determined the k_{cat} and K_M values for a broad range of acetyl esters with leaving group exhibiting pK_a values in the range of 7−17. These include seventeen aryl esters (Table 2, compounds **16**−**31** and **33**), two benzylic esters (benzyl acetate (**37**) and 1-phenylvinyl acetate (**32**)), and several aliphatic acetate esters (**34**−**36** and **38**−**41**).

The plots of both log(k_{cat}) and log(k_{cat}/K_M) versus leaving group pK_a (Figure 2, parts A and B, respectively), show that, for aryl esters with small substituents at meta and ortho positions (fluoro-substituted aryl esters and phenyl acetate), there is almost no dependence of rate on leaving group pK_a (Figure 2, full squares). The kinetic parameters for phenyl acetate ((**29**), pK_a = 10) are very similar to 2,6-difluorophenyl acetate ((**19**), pK_a = 7.3, Table 2). If anything, the rates go down slightly as the leaving group becomes more acidic. A large number of aromatic ester substrates fall under this plateau rate. In general, PON1 is much less reactive toward substrates with large substituents at the meta and para positions (Figure 2, empty circles). Substituents at meta positions have a smaller inhibitory effect than substituents at para positions. For example, the k_{cat} of 3-nitrophenyl acetate is 59 s^{-1} (**22**), and the k_{cat} of 4-nitrophenyl acetate (**16**) is 26 s^{-1} (relative to phenyl acetate (**29**) with a k_{cat} of about 700 s^{-1}). The same effect is observed with cyano substituents, the k_{cat} of 3-cyanophenyl acetate (**25**) is 125 s^{-1}, while the k_{cat} of 4-cyanophenyl acetate (**20**) is 23 s^{-1}. Because the pK_a values of all these *para*-phenols are lower than the corresponding *meta*-phenols, these variations may indicate a rather unusual positive β_{LG} value (β > 0). However, the reductions in rates correlate better with the size of the substituents rather than their electron withdrawing abilities. We also suspected that para substituents containing oxygen exhibit low rates due to a nonproductive binding mode (e.g., by the subsitituent interacting with the catalytic calcium instead of the ester). Indeed, among the aryl esters, the lowest activity is observed with *p*-acetoxy acetophenone ((**21**); k_{cat} = 13 s^{-1}) and *p*-acetoxy methyl benzoate ((**24**), k_{cat} = 14 s^{-1}). However, the low rates observed with 3,4-dimethylphenol acetate ((**33**), k_{cat} = 51 s^{-1}) strongly argue for a pure meta/para steric effect. As is the case with the aryl phosphotriesters (Figure 1), the log(k_{cat}) and log(k_{cat}/K_M) plots (Figure 2, parts A and B, respectively) provide a very similar picture. This is the result of the K_M values for all aryl acetate substrates being in the range of 2 ± 1 mM, with minor exceptions (substrates **20** and **31**, K_M > 4 mM).

As the leaving group pK_a increases above 10.5, the rates of catalysis go down dramatically. Nevertheless, a measurable rate was observed with alkyl esters in contrast to previous reports (*17*). The hydrolysis of aliphatic acetate esters (pK_a 12.5−16.1) is obviously much slower than the hydrolysis of phenyl acetate. However,the k_{cat} for the hydrolysis of trifluoroethyl acetate ((**34**), pK_a = 12.5) is higher than the k_{cat} for 1-phenylvinyl acetate hydrolysis ((**32**), pK_a = 10.3), and the k_{cat} values of butyl acetate and propyl acetate (pK_a ∼ 16.1) are higher that the k_{cat} of benzyl acetate (pK_a = 15.3). The steric effects are probably as pronounced as the electronic ones, and the low rates of the two benzylic esters (**32** and **37**) are probably due to steric hindrance by benzylic substituent. This may be partly reflected in the slightly higher K_M values of benzylic esters (∼5 mM).

The choice of aliphatic leaving groups is much more restricted in pK_a than the aromatic ones are. This makes any determination of the β_{LG} value for the aliphatic esters problematic. A line based on ethyl acetate and its fluorinated analogues (compounds **34**−**36** and **38**) would give a slope

Table 2: Kinetic Parameters for Esters Hydrolysis by rePON1 G2E6

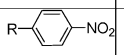
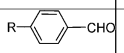
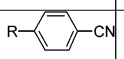
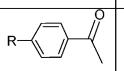
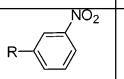
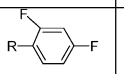
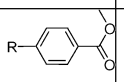
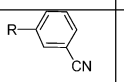
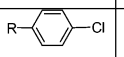
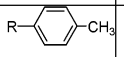
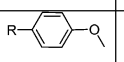
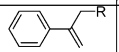
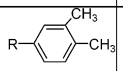
compound	**Formula R=acetoxy** 	**pKa**	**λmax nm**	**Є OD/M**[a]	**k_{cat} s^{-1}**	**K_M mM**	**k_{cat}/K_M s^{-1} M^{-1}**
4-nitrophenyl acetate [**16**]		7.14[b]	405	10,510	26 ± 2	1.5 ± 0.2	17,000 ± 1,400
2,6 difluoro-phenyl acetate [**17**]		7.30[b]	270	950	145 ± 9	2.0 ± 0.5	77,000 ± 16,000
4-acetoxy benzaldehyde [**18**]		7.66[b]	330	12,620	26 ± 2	2.3 ± 0.2	11,100 ± 800
2,3-difluoro-phenyl acetate [**19**]		7.81[b]	275	606	403 ± 26	1.19 ± 0.05	340,000 ± 24,000
4-cyanophenyl acetate [**20**]		7.95[b]	275	7,495	~23	~4	~5,300 Linear fit[c]: 3,600 ± 280
4-acetoxy acetophenone [**21**]		8.05[b]	320	8,080	13 ± 1	2.6 ± 0. 3	5,100 ± 560
3-nitrophenyl acetate [**22**]		8.39[b]	340	840	59 ± 4	2.2 ± 0.1	26,000 ± 1,000
2,4-difluoro-phenyl acetate [**23**]		8.43[b]	275	920	334 ± 19	2.23 ± 0.3	150,000 ± 12,800
4-acetoxy methyl benzoate [**24**]		8.47[b]	295	5485	~14	~2	~5,700 Linear fit[c]: 6,000 ± 150
3-cyanophenyl acetate [**25**]		8.61[b]	295	1,610	125 ± 15	1.9 ± 0.4	68,000 ± 9,400
2-fluorophenyl acetate [**26**]		8.81[b]	270	710	431 ± 11	1.50 ± 0.2	295,000 ± 31,000
3-fluorophenyl acetate [**27**]		9.28[b]	270	656	571 ± 18	1.4 ± 0.2	410,000 ± 52,000
4-chlorophenyl acetate [**28**]		9.38[b]	280	848	117 ± 2	1.0 ± 0.1	114,000 ± 14,000
Phenyl acetate [**29**][d]		10.00[e]	270	700	698 ± 26	1.2 ± 0.2	595,000 ± 100,000
4-methyl phenyl acetate [**30**]		10.20[f]	276	1,520	68 ± 7	1.26± 0.15	54,000 ± 1,300
4-methoxy phenyl acetate [**31**]		10.29[e]	290	1,254	39 ± 2	4.1 ± 0.3	9,500 ± 170

Table 2. (Continued)

compound	Formula R=acetoxy 	pKa	λmax nm	€ OD/M[a]	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M s^{-1} M^{-1}
1-phenylvinyl acetate [**32**]		10.34[g]	pH indicator assay		~1	~5	~270 Linear fit[c]: 201 ± 8
3,4 dimethyl phenyl acetate (**33**)		10.36[h]	276	850	51 ± 2	2.1 ± 0.3	25,000 ± 3,000
Trifluoroethyl acetate [**34**]		12.4[f]	pH indicator assay		~4	~19	~220 Linear fit[c]: 182 ± 4
2,2 difluoroethyl acetate [**35**]		13.3[f]	pH indicator assay		0.82 ± 0.07	24.8 ± 3.6	33 ± 2
2-fluoroethyl acetate [**36**]		14.2[f]	pH indicator assay		~0.5	~54	~9 Linear fit[c]: 7.5 ± 0.9
Benzyl acetate [**37**]		15.2[i]	pH indicator assay		~0.02	~5	~4 Linear fit[c]: 4.3 ± 0.4
Ethyl acetate [**38**]		16.1[j]	pH indicator assay		0.242 ± 0.007	15.4 ± 0.5	15.8 ± 0.5
Propyl acetate [**39**]		16.1[j]	pH indicator assay		0.400 ± 0.009	13 ± 1	30 ± 2
Butyl acetate [**40**]		16.1[j]	pH indicator assay		0.27 ± 0.01	10 ± 2	27 ± 5
Isopropyl acetate [**41**]		17.1[j]	pH indicator assay		Non-detectable activity		

[a] The λ_{max} and extinction coefficients of the respective substituted phenols products were determined spectrophotometrically at pH 8.0 on a microtiter plate reader (PowerWave HT microplate scanning spectrophotometer; optical length, ~0.5 cm). [b] From ref *22*. [c] An appropriate fit to the Michaelis−Menten equation was not possible due to limited substrate solubility (maximal substrate concentration was $<2 \times K_M$). The approximate values are the values obtained from fit to the Michaelis−Menten equation [$v_0 = k_{cat}[E]_0[S]_0/([S]_0 + K_M)$], and the accurate k_{cat}/K_M values were obtained from a linear fit in the pseudo-second-order region of the Michaelis−Menten model [$v_0 = [S]_0[E]_0k_{cat}/K_M$]. [d] The kinetic parameters previously reported by our group ($k_{cat} = 965\ s^{-1}$, $K_M = 0.43$ mM, and $k_{cat}/K_M = 2.2 \times 10^6\ M^{-1}\ s^{-1}$, (*10*)) were obtained using phenyl acetate stocks in DMSO without equalizing the solvent percentage in all the substrate concentrations. [e] From ref *31*. [f] From ref *23*. [g] From ref *27*. [h] From ref *20*. [i] From ref *28*. [j] From ref *26*.

of −0.30 ($R = 0.90$). Notably, the slope connecting the two benzylic compounds (**32** and **35**, pK_a leaving group 10.34 and 15.2, respectively) is quite similar (−0.37), although the absolute rates are much lower. The K_M values of aliphatic esters are generally higher than those of phenolic and benzylic esters and range from 10 to 54 mM. But the overall picture remains the same with both k_{cat} and k_{cat}/K_M plots.

In summary, a completely different pattern is observed for aryl versus aliphatic esters. Steric hindrance put aside, aryl esters show quite high rates, with a k_{cat} of almost 1000 s^{-1} and k_{cat}/K_M values that are close to $10^6\ M^{-1}\ s^{-1}$. The absence of dependence on leaving group pK_a, suggests that these rates are limited by a physical step, such as product release, or a conformational change and not by a chemical barrier (transition state stabilization or bond breaking involving the leaving group). In contrast, the aliphatic esters show a sensitivity to leaving group pK_a that is similar to the hydroxide-catalyzed, nonenzymatic hydrolysis in solution ($\beta_{LG} = -0.3$) (*35*), and the overall rates are far slower than expected for a native substrate ($k_{cat} < 1\ s^{-1}$ and $k_{cat}/K_M < 10^2\ M^{-1}\ s^{-1}$, for nonactivated aliphatic esters such as **38**−**40**).

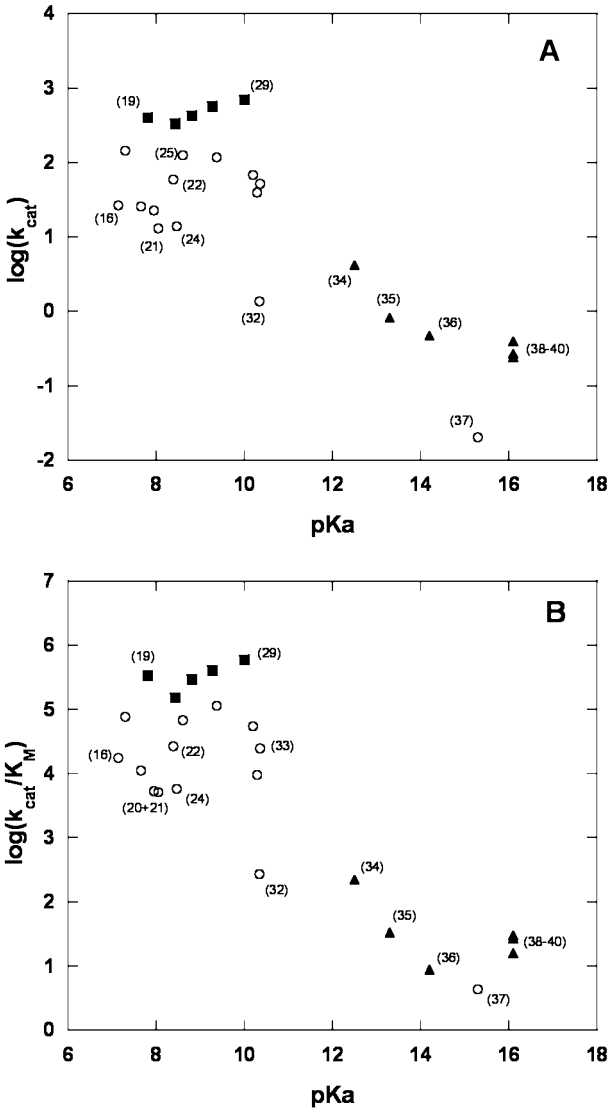


FIGURE 2: Structure−activity relationships for the hydrolysis of esters by rePON1. Plotted are the logarithmic values of k_{cat} (A) and k_{cat}/K_M (B) vs pK_a of the leaving group. Aryl ester substrates with small meta and ortho substituents are shown in full squares, and their rates seem to be largely independent of leaving group pK_a ($\beta_{LG} \sim 0$). Other aryl esters and benzyl esters (empty circles) given much lower rates. Esters with aliphatic leaving groups are denotated as full triangles. Numbers refer to the substrates entry in Table 2.

The Lactonase Activity of PON1. The kinetic parameters k_{cat} and K_M were determined for hydrolysis of dihydrocoumarin and several aliphatic γ-lactones and δ-lactones with side chains of various lengths. The results (Table 3) show that there is virtually no dependence of the kinetic parameters on the leaving group pK_a, since the rate of hydrolysis of dihydrocoumarin (a phenol with $pK_a^{LG} \sim 10$) is comparable to the rate of hydrolysis of γ-butyrolactone and δ-valerolactone ($pK_a^{LG} \sim 16$, of aliphatic alcohols). The rate of hydrolysis of aliphatic lactones is substantially higher than the rate of hydrolysis of their noncyclic ester analogues. For example, the k_{cat} for δ-valerolactone is >500-fold faster than those of ethyl, propyl, and butyl acetate. The differences are much more pronounced in k_{cat}/K_M values, which are higher by ~23 000-fold for lactones than those for ethyl, propyl, and butyl acetate. This is due to large variations in the K_M values of these substrates. Interestingly, while the K_M values of all phosphotriester and aryl ester substrates are very similar, the K_M values for lactones differ dramatically, from 0.129 mM up to 21mM. PON1 appears to be sensitive to the size of the lactone ring (K_M for γ-butyrolactone (**42**) is 21 mM, and that of and δ-valerolactone (**49**) is 0.57 mM), as well as the ring substituents (e.g., **42** versus **44**).

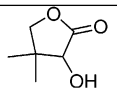
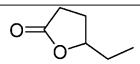
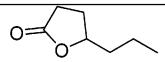
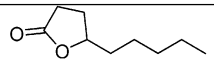
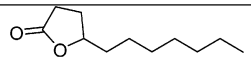
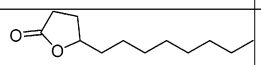
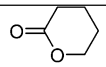
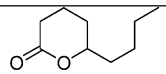
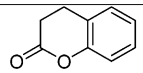
Inhibition Studies. A substrate range as broad as PON1's is not common. We therefore sought support for the notion that the hydrolysis of all the substrates discussed above takes place in the same active site. We applied 2-hydroxyquinoline, a known inhibitor of PON1 (*10*), for the inhibition of the lactone substrate δ-valerolactone and the most familiar substrates of PON1, paraoxon, and phenyl acetate. We found that 2-hydroxyquinoline is a competitive inhibitor of all these substrates, as indicated by the intersecting double-reciprocal plots (Figure 3). All the examined substrates have very similar inhibition constants 0.9−2.7 μM (Supporting Information Table 4). This suggests that all substrates are hydrolyzed at the same active site, but it does not obviously indicate that all substrates are positioned in the same manner. On the contrary, it appears that different substrates occupy different subsites within the same active site and make use of different catalytic residues (*36*), but their binding sites overlap that of the inhibitor to a degree that prevents mutual binding.

DISCUSSION

General Considerations. SAR studies are useful in dissecting enzyme mechanisms and, in particular, in understanding how the active site and mechanism of a given enzyme are tailored for its natural substrate and reaction. In our interpretation of the LFER study results, we assumed that the enzymatic and nonenzymatic hydrolysis reactions proceed by the same mechanism (that is, through similar transition states). Viscosity experiments (Supporting Information , part E) demonstrated that the hydrolysis of neither paraoxon nor phenyl acetate (which is one of the best substrates of PON1) is diffusion-controlled. This implies that the rate-determining step of the enzymatic reaction is the chemical step. We would therefore like to analyze the results presented above in view of this question: what is the activity for which PON1's active site is tailored?

A basic assumption underlines this analysis: steric considerations need to be excluded as, apart from lactones **42**−**50**, all other substrate tested are man-made chemicals of no physiological relevance and the level to which PON1's active site accommodates them is therefore irrelevant. Our analysis is therefore based primarily on those substrates where the rate appears to be affected by leaving group pK_a only. Two major considerations apply to these substrates. First, the β_{LG} value, namely, how the enzymatic rate of hydrolysis changes as the leaving group pK_a increases. It is assumed that a higher (less negative) β_{LG} value for the enzyme-catalyzed reaction in relation to nonenzymatic, hydroxide-catalyzed reaction may indicate that the active site is tailored to the reaction's transition state. For example, an enzymatic β_{LG} value close to zero, as oppose to, say, −0.6 for solution, is indicative of an active-site general-acid residue that protonates the leaving group and significantly facilitates bond breakage (*21*) or a metal-catalyzed reaction, in which a positive metal ion stabilizes the negative charge formed on the leaving group.

Table 3: Kinetic Parameters for Lactones Hydrolysis by rePON1 G2E6

name	structure	pKa	k_{cat} s^{-1}	K_M mM	k_{cat}/K_M s^{-1} M^{-1}
γ-butyrolactone [**42**]		~16[a]	~111	~21	~5300 Linear fit [b]: 3800 ± 120
Pantoyl lactone (racemic) [**43**]		~16[a]	11.9 ± 0.4	4.3 ± 0.6	2800 ± 350
γ –caprolactone [**44**]		~17[a]	44 ± 1	1.0 ± 0.1	43,000 ± 6,700
γ –heptanolide [**45**]		~17[a]	34.0 ± 0.8	0.58 ± 0.03	58,000 ± 3,000
γ -nonanoic lactone [**46**]		~17[a]	31 ± 2	0.39 ± 0.03	78,000 ± 1,600
γ-undecanoic lactone [**47**]		~17[a]	62 ± 2	0.60 ± 0.07	103,000 ± 8,600
γ-dodecanoic lactone [**48**]		~17[a]	26.4 ± 0.4	0.22 ± 0.02	123,000 ± 10,000
δ-valerolactone [**49**]		~16[a]	210 ± 9	0.57 ± 0.05	370,000 ± 35,000
δ-nonalactone [**50**]		~17[a]	48 ± 2	0.812 ± 0.003	60,000 ± 2,200
Dihydrocoumarin [**51**]		~10[c]	152 ± 3	0.129 ± 0.008	1,190,000 ± 49500

[a] From ref *26*. [b] An appropriate fit to the Michaelis−Menten equation was not possible due to a solubility problem (maximal substrate concentration was below $2-3 \times K_M$). The approximate values are the values obtained from fit to the Michaelis−Menten equation, and the accurate k_{cat}/K_M values were obtained from the linear fit in the low-substrate concentration region of the Michaelis−Menten curve. [c] From ref *31*.

In contrast, an enzymatic β_{LG} value that is more negative than that of the nonenzymatic reaction, suggests that the active site is not tailored for this type of reaction or substrate and that the negative charge that accumulates on the leaving group as the transition state is approached is not well tolerated (*22*). The active site of PON1 is hydrophobic, and one could argue that it might a priori be unsuitable for stabilizing charged transition states. However, there are enzymes with hydrophobic active sites and hydrophobic native substrates that can tolerate the negative charge that accumulates on the leaving group, for example, by utilizing general-acid catalysis (*23*). The second criterion is the turnover rates (k_{cat}), rate acceleration, and catalytic efficiency, in particular (k_{cat}/K_M). Enzymes show k_{cat}/K_M values toward their native substrates that are in the range 10^6-10^8 M^{-1} s^{-1}. The turnover rates (k_{cat}) of enzymes vary over many orders of magnitude (from >1 min^{-1} up to 10^6 s^{-1}). The k_{cat} value is a function of physiological necessity (the need to generate a certain number of product molecules per second) and of the nonenzymatic rate (k_{uncat}). Hence, the lowest k_{cat} values are usually seen with reactions for which the spontaneous rates are extremely slow (half-lives of millions, if not billions, of years) (*37*). Esterases and phosphotriesterases exhibit k_{cat} values that are generally above >50 s^{-1}, while rates of $\sim 10^4$ s^{-1} are also documented (*38*, *39*).

PON1 as a Phosphotriesterase. Paraoxon, from which PON1 takes its name, is a synthetic compound that appeared on Earth several decades ago. It is therefore unlikely that PON1 evolved to hydrolyze this substrate specifically. It may, however, have evolved as a phosphotriesterase for phosphotriester substrates other than paraoxon. Is PON1 a phosphotriesterase? Given the above criteria, the answer would be no. First, the β_{LG} value for PON1-catalyzed phosphotriester hydrolysis is ca. −1.6 (for leaving group pK_a values >7.2), whereas the β_{LG} value for alkaline hydrolysis of phosphotriesters is −0.44 (*29*). Interestingly, a similar behavior is observed with the bacterial phosphotriesterase (PTE), for which paraoxon is thought to be the native substrate (*29*) and where the rates for substrates with leaving group pK_a > 7.2 exhibit a highly negative β_{LG} value (< −2). This is explained by the fact that this enzyme might have evolved specifically for paraoxon that has a leaving group pK_a around neutrality (7.14) and therefore possesses no mechanism for stabilization of the leaving group's negative charge. Indeed, as is the case of paraoxon, the rates for paraoxon and substrates with leaving group pK_a < 7 are at plateau. Yet, the difference between bacterial PTE and PON1 regards the second consideration, namely, rates. PTE's rates for paraoxon hydrolysis are impressively high ($k_{cat} \geq 2 \times 10^3$ s^{-1} and $k_{cat}/K_M > 10^7$ M^{-1} s^{-1}) (*38*). However, PON1's rates at the plateau region are much lower: $k_{cat} \approx 10$ s^{-1}, and $k_{cat}/K_M < 10^4$ M^{-1} s^{-1}. Thus, both criteria, β_{LG} values and rates, are inconsistent with PON1 being a phosphotriesterase, at least not for aryl diethyl phosphates such as paraoxon.

Is PON1 an Esterase? The SAR results indicate a completely different pattern for aryl versus aliphatic esters. Steric consideration put aside, the aryl esters show a plateau of rates that are largely independent of leaving group pK_a

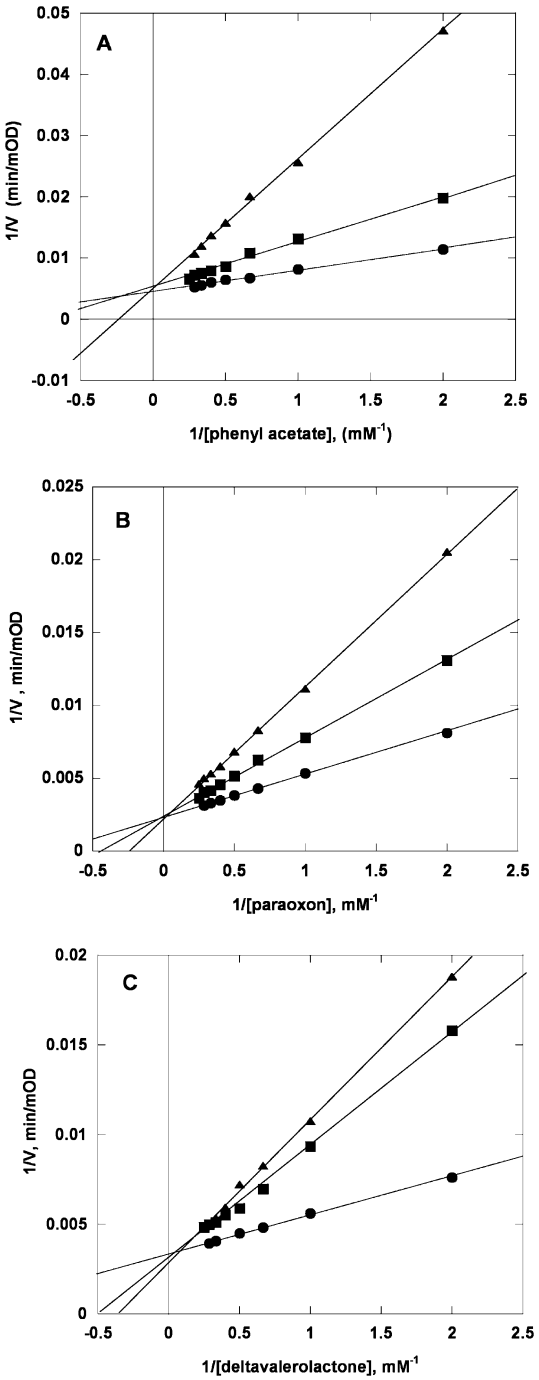


FIGURE 3: Competitive inhibition of hydrolytic activities of rePON1 by 2-hydroxyquinoline. Shown are double reciprocal plots of initial velocities vs substrate concentrations. (A) Phenyl acetate as substrate, at 0 (circles), 1 (rectangles), and 6 μM (triangles) 2-hydroxyquinoline. (B) Paraoxon as substrate, at 0 (circles), 2 (squares), and 6 μM (triangles) 2-hydroxyquinoline. (C) δ-Valerolactone concentrations as substrate, at 0 (circles), 2 (squares), and 5 μM (triangles) 2-hydroxyquinoline. Fitting the data to competitive inhibition equation gave the following K_i values: 0.9 $\pm$ 0.2 μM for phenyl acetate, 2.7 $\pm$ 0.7 μM for paraoxon, and 1.42 $\pm$ 0.02 μM for δ-valerolactone.

and level off at a k_{cat} of almost 1000 s^{-1} and k_{cat}/K_M values that are close to 10^6 M^{-1} s^{-1}. It appears that the rates of catalysis of aryl esters are quite high and may be limited by a physical step other than substrate binding, for example, product release, or a conformational change and not by a chemical step such as bond-breaking. There is also a possibility that the rate-determining step in ester hydrolysis is a breakdown of a covalent acyl−enzyme intermediate, although bursts of product release were not seen with aryl esters or any other substrate. In contrast, the aliphatic esters show a sensitivity to leaving group pK_a that is similar to the hydroxide-catalyzed, nonenzymatic hydrolysis in solution ($\beta_{LG} \sim -0.3$), and the overall rates are far slower than expected for a native substrate ($k_{cat} < 1$ s^{-1}; $k_{cat}/K_M \ll 10^2$ M^{-1} s^{-1}). Thus, considering both β-values and rates, it appears that PON1 may well be an aryl-esterase but not a broad-range esterase that hydrolyses aliphatic esters or even benzylic esters.

Is PON1 a Lactonase? Judging by β-values as well as rates, the answer appears to be yes. The rate of hydrolysis of δ-valerolactone (**49**) with an aliphatic alkoxy leaving group of p$K_a \sim 16$ is similar to that of dihydrocoumarin (**51**) with a phenolic leaving group pK_a of ~10. It therefore appears that the rates of lactone hydrolysis are independent of leaving group pK_a ($\beta_{LG} \approx 0$), although this conclusion could not be further substantiated due to the unavailability of lactones with leaving group pK_a values in the range between 10 and 16. The rates are high in absolute terms: a range of aliphatic lactones exhibits k_{cat} values of 30−210 s^{-1} and k_{cat}/K_M values of 10^5−10^6 M^{-1} s^{-1}. The differences in rates between lactones and their respective noncyclic, or "open", ester analogues are very large: k_{cat} for δ-valerolactone is >500 times faster than that of propyl and butyl acetate, and k_{cat}/K_M is ~10^4 times higher. These differences can be attributed, in part, to the intrinsic higher reactivity of lactones versus noncyclic esters, since the nonenzymatic, hydroxide-catalyzed hydrolysis of five-membered ring lactones was found to be 300 times faster than the hydrolysis of their open-chain analogues, and acceleration factors of up to 5500 were observed with six-membered rings (*40*). However, the enzymatic hydrolysis of lactones by PON1 does not correlate directly with intrinsic reactivity, as five- and six-membered ring lactones exhibit similar k_{cat} values, while in solution, the latter are >15 times more reactive (*40*). As discussed below, the differences between cyclic and open esters are not only in k_{cat} but also in K_M.

The Substrate Binding Mode(s). Can we learn something from the K_M values of the various types of substrates? The schematic view of enzyme catalysis is that the energetics of substrate binding are reflected in the K_M and the catalysis by k_{cat}. In the simplistic case, where $K_M \approx K_S$, one would expect substrates that bind poorly due to steric hindrance, for example, to exhibit high K_M values. However, all PON1's aryl ester and phosphotriester substrates, whether extremely poor ($k_{cat}/K_M < 1$ M^{-1} s^{-1}) or very effective ($k_{cat}/K_M > 10^5$ M^{-1} s^{-1}), exhibit K_M values in the 1−4 mM range. The differences in reactivity are primarily due to very different k_{cat} values (Tables 1 and 2). This also applies to cases where the poor rates are clearly the result of steric hindrance, namely, to all phenyl phosphotriesters with ortho substituents (Figure 1, empty circles), as well as phenyl esters with para substituents (Figure 2, empty circles). A plausible explanation is that, for all the aryl ester and phosphotriester substrates, substrate binding is driven primarily by nonspecific hydrophobic forces to the very deep and narrow hydrophobic active site of PON1 (*14*). This conclusion is further supported by an approximate correlation ($R = 0.84$) we observed between substrate hydrophobicity and K_M values: the less hydrophobic the substrate is (low log P values), the higher the K_M

value (Supporting Information, Figure 1). It appears that, for PON1, the mode of binding differs, as the poor substrates are inadequately positioned relative to the catalytic machinery, and hence exhibit very low k_{cat} values.

The lactones are the only substrate type where K_M values vary over more than 2 orders of magnitude (0.129−21 mM, Table 3), while the variations in k_{cat} values are quite modest (12−210 s^{-1}). A clear difference is also seen between the K_M values of most lactones that are in the range of 0.5 mM (**45**−**50**) and the corresponding open esters (**38**−**40**) that are >10 mM. These differences could be attributed to the higher intrinsic reactivity of lactones (e.g., a covalent acyl−enzyme intermediate and a change in rate-limiting step from acylation in open esters to deacylation in lactones) (*39*). But, not all lactones exhibit low K_M values (e.g., **42**, **43**). Further, PON1 appears to be sensitive to the size of the lactone ring (the K_M for γ-butyrolactone (**42**, 21 mM) is 37 times higher than that of δ-valerolactone (**49**, 0.57 mM)), as well as to the ring substituents (e.g., **42** vs **44**). Binding of lactones appears to be driven by specific active-site interactions, and substrates that fit poorly exhibit high K_M values (low affinity) yet reasonable k_{cat} values. Thus, a clear difference is seen in the mode of binding of lactone substrates that appears to be specific versus the nonspecific mode of binding of esters (and aryl esters in particular) and phosphotriesters.

Concluding Remarks. PON1 hydrolyzes many types of substrates: phosphotriesters, esters, and lactones. Inhibition studies with the competitive inhibitor 2-hydroxyquinoline suggest that all these types of PON1 substrates are hydrolyzed at the same active site. The active site calcium ion is likely to serve as a common theme by stabilizing the oxyanionic intermediates and transition states on route to hydrolysis of all these substrates. The differences between the substrates are probably due to different modes of binding (positioning, orientation) of the substrate in the active site and to differences in the rate-determining steps. This may be reflected in the fact that both aryl esters and aryl phosphotriesters show a plateau of rates under a certain pK_a which is consistent with a rate determining product release, or conformational change, but the rates differ; k_{cat} plateau for aryl esters is ~1000 s^{-1}, and that of aryl phosphotriesters is ~10 s^{-1}. Since we have not observed burst kinetics with any substrate, it is conceivable that the hydrolysis does not proceed via a covalent acyl−enzyme or phosphoryl−enzyme intermediate and is general-base catalyzed in all cases. Yet, a different active-site residue may act as a base for the different types of substrates (*36*). These mechanistic details are still enigmatic and have to be studied further. There are, however, several mechanistic insights that can be deduced from this study and may serve as a basis for further exploration of PON1's mechanism of action and biological role. Foremost is the conclusion that PON1 is not a paraoxonase nor an esterase but a lactonase. This conclusion is very much in line with recent findings that PON1 exhibits lactonase, as well as lactonizing, activity with metabolites of fatty acid oxidation (*17*, *18*). PON1 was also found to exhibit a low, but distinct, sequence similarity with a lactonohydrolase from *Fusarium oxysporum* (*41*). The main substrate of this enzyme is D-pantolactone, which is also hydrolyzed by PON1, although at low efficiency (**43**, $k_{cat}/K_M \approx 3000$ M^{-1} s^{-1}). In addition, directed evolution experiments indicated that PON1 variants selected for improved esterase or phosphotriesterase activities exhibit dramatic changes in these activities (including with substrates that were not selected for) but largely retain their lactonase activity. The robustness of the lactonase activity is a characteristic of the native function, while the promiscuous esterase and phosphotriesterase activities respond abruptly to mutations (*42*). A very similar trend is seen in the nature—the common denominator of all PON subfamilies (PON1, 2, and 3) is their lactonase activity (*1*), whereas the other activities are sporadic (e.g., PON2 and PON3 exhibit no, or barely detectable, phosphotriesterase activity) (*1*, *10*).

Thus, although the very first identification of PON1 might have been as a calcium-dependent serum lactonase (*43*), it was later rediscovered as an aryl esterase and then as paraoxonase (*1*). It is now clear that all three activities reside in the same active site and that this enzyme is most likely a lactonase.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information containing synthesis procedures, identification of the substrates, cosolvent effects, correlation between kinetic parameters of esters hydrolysis and hydrophobicity coefficients, and competitive inhibition data. This material is available free of charge via the Internet at http://pubs.acs.org.

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