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The Histidine 115-Histidine 134 Dyad Mediates the Lactonase Activity of Mammalian Serum Paraoxonases*

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Serum paraoxonases (PONs) are calcium-dependent lactonases that catalyze the hydrolysis and formation of a variety of lactones, with a clear preference for lipophilic lactones. However, the lactonase mechanism of mammalian PON1, a high density lipoprotein-associated enzyme that is the most studied family member, remains unclear, and other family members have not been examined at all. We present a kinetic and site-directed mutagenesis study aimed at deciphering the lactonase mechanism of PON1 and PON3. The pH-rate profile determined for the lactonase activity of PON1 indicated an apparent pK_a of ~7.4. We thus explored the role of all amino acids in the PON1 active site that are not directly ligated to the catalytic calcium and that possess an imidazolyl or carboxyl side chain (His^{115}, His^{134}, His^{184}, His^{285}, Asp^{183}, and Asp^{269}). Extensive site-directed mutagenesis studies in which each amino acid candidate was replaced with all other 19 amino acids were conducted to identify the residue(s) that mediate the lactonase activity of PONs. The results indicate that the lactonase activity of PON1 and PON3 and the esterase activity of PON1 are mediated by the His^{115}-His^{134} dyad. Notably, the phosphotriesterase activity of PON1, which is a promiscuous activity of this enzyme, is mediated by other residues. To our knowledge, this is one of few examples of a histidine dyad in enzyme active sites and the first example of a hydrolytic enzyme with such a dyad.

Serum paraoxonases (PONs)[2] constitute a family of calcium-dependent mammalian enzymes that have been recently defined as lipophilic lactonases. PON1 is the best studied member of the family, with other members being PON2 and PON3 (1, 2). PON1 catalyzes the hydrolysis of multiple substrates: lactones, thiolactones, carbonates, esters, and phosphotriesters, including paraoxon, from which its name is derived. However, only after a few decades of research, it became apparent that PON1 and the other PONs are in fact lactonases (3–6), catalyzing both the hydrolysis (4, 6) and formation (7) of a variety of lactones. Structure-reactivity studies (6) and laboratory evolution experiments (3) indicate that the native activity of PON1 is lactonase. The other activities, *e.g.* arylesterase and phosphotriesterase (paraoxonase), are merely promiscuous and are not shared by other family members, *e.g.* PON2 and PON3. PON1 activation by binding to high density lipoprotein particles carrying apoA-I also indicates high specificity toward lactone substrates and, in particular, lipophilic lactones that display k_{cat}/K_m values of 10^6–10^7 M^{-1} s^{-1} (5). The physiological substrates of PONs are still unknown, but they are likely to include lactones consumed as food ingredients (8) or derivatives of fatty acid oxidation processes, *e.g.* 5-hydroxyeicosatetraenoic acid lactone (4, 8), that reside in high density lipoprotein, low density lipoprotein, or macrophage cells.

PON1 is composed of 354 amino acids. The enzyme has two calcium-binding sites: the higher affinity calcium is required for the structural integrity, whereas the lower affinity calcium is involved in catalysis (9). Early mechanistic studies using chemical labeling and site-directed mutagenesis identified several residues that are involved in the phosphotriesterase and esterase activities of human PON1 (10, 11). However, because these studies were conducted before the three-dimensional structure of PON1 was known, it was largely unclear whether these amino acids are indeed in the PON1 active site or whether they are involved in substrate binding, Ca^{2+} binding, or catalysis.

Recently, a crystal structure of a recombinant PON1 (rePON1) variant (G2E6) was solved at a resolution of 2.2Å, providing the first structure of a PON family member (12). This variant was directly evolved from rabbit PON1. It is expressed in a soluble and active form in *Escherichia coli* and exhibits enzymatic properties that are essentially identical to those reported for PON1 purified from sera. PON1 was found to be a six-bladed β-propeller, with the two calcium ions located in the central tunnel. The structural calcium (Ca2) is buried, whereas the catalytic calcium (Ca1) is solvent-exposed and located at the bottom of a deep hydrophobic active site. The active-site residues of PON1 were also defined by amino acids whose alteration during directed evolution shifted the activity and substrate selectivity of PON1.

The structure of PON1 allowed us to postulate its mechanism of catalysis (12). On the basis of pH-rate profiles constructed for paraoxon and 2-naphthyl acetate hydrolysis, an unprotonated histidine was supposed to be involved in the base-catalyzed rate-determining step of catalysis by PON1. A histidine dyad composed of His^{115} and His^{134} was suggested to be directly involved in the catalytic mechanism of PON1 for both ester and phosphotriester hydrolysis. Mutagenesis experiments supported the suggested mechanism, although it was later found that these mutants were probably misfolded and therefore inactive (13). Moreover, Yeung *et al.* (14) recently reported that the H115W mutant of human PON1 retains activity with paraoxon. They therefore postulated that His^{115} is important for substrate binding and specificity, but does not directly participate in catalysis (15).

Most important, all previous mechanistic studies of PON1 addressed the phosphotriesterase and esterase activities, but the mechanism of lactone hydrolysis, which now appears to be the primary function of PON1, was not explored. The mechanism of other mammalian PON family members, most notably PON3, which exhibits weak esterase activity and almost no paraoxonase activity, has not been studied either.

This study aimed to decipher the lactonase mechanism of PON1 and PON3. We determined the pH-rate profile for lactone hydrolysis and conducted extensive site-directed mutagenesis studies to identify the residues that mediate this activity. We show that the lactonase and

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[2] The abbreviations used are: PONs, serum paraoxonases; rePON1, recombinant PON1; TBBL, 5-(thiobutyl)butyrolactone; MES, 4-morpholineethanesulfonic acid; bis-tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane.

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esterase activities of PON1 are mediated by the His[115]-His[134] dyad and rule out other active-site residues, including His[285]. Finally, the accompanying article (16) shows that the PON1 mutants with reduced lactonase activity studied here (H115Q, H134Q, and the double mutant H115Q/H134Q) exhibit reduced or no biological function in *ex vivo* assay. We also show that the paraoxonase activity is promiscuous in terms of substrate binding, but is also mediated by residues other than those that mediate the native activity.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Aldrich, Fluka, and Acros Organics. Primers for site-directed mutagenesis were purchased from Sigma.

Site-directed Mutagenesis—The pET32b(+) plasmid containing the gene for rePON1-G2E6 (12) was used as a template for PCR amplification. The mutants were constructed by the "inverse PCR" method using two neighboring non-overlapping primers, one of which bears the mutation at its 5′-end (17). *Pfu* Turbo polymerase was applied for 25 cycles of polymerization at 72 °C. After digestion of the template plasmid with DpnI, the amplified DNA was blunt-ligated with T4 ligase, and the ligated DNA was transformed into *E. coli* DH5α cells. The mutated genes were verified by DNA sequencing. The histidine replacement libraries were generated by replacing His codons with DNS codons (where D is an equimolar mixture of A, G, and T; N is a mixture of all four nucleotides; and S is a mixture of C and G) encoding all amino acids except His, Gln, and Pro. The Gln replacement libraries were generated separately by replacing the His codon with a Gln codon (CAG). The aspartate replacement libraries were produced by introducing a combination of NNR and HNS degeneracy codons (where R is an equimolar mixture of A and G, and H is an equimolar mixture of A, C, and T) encoding all amino acids except Asp.

Screening of rePON1 Mutants—The libraries were transformed into *E. coli* Origami B DE3 cells (Novagen). Colonies grown on agar were used to inoculate 500 μl of LB medium in a 96-well plate and were grown overnight at 37 °C. The plates were duplicated; lysed with Bug-Buster (Novagen); and screened for esterase (2-naphthyl acetate; 0.2 mM), phosphotriesterase (paraoxon; 0.5 mM), and lactonase (5-(thiobutyl)butyrolactone (TBBL) (18); 0.22 mM) activities.

Expression and Purification of rePON1-G2E6 Mutants—Wild type-like rePON1-G2E6 and its various mutants were expressed as fusion proteins with thioredoxin and His_6 tags and purified as described previously (19), except that mutant proteins were eluted from a nickel-nitrilotriacetic acid column in buffer containing 10% glycerol, 0.1% Tergitol, and 50 mM NaCl. The purity of wild-type rePON1 and its mutants was analyzed by SDS-PAGE, and the proteins were essentially pure (>90%). The expression levels of wild-type rePON1 and the purified mutants were 20–50 mg/liter of culture.

pH-rate Profile—k_{cat} and K_m values were determined for rePON1-G2E6 with TBBL (18) at pH 5.8–9.4. Initial velocities (v_0) were determined at eight different concentrations for each substrate. The buffers used were MES (pH 5.8–6.5) and bis-tris propane (pH 6.5–9.4) at 0.1 M plus 1 mM $CaCl_2$. The ionic strength was adjusted to a total of 0.2 M with NaCl. The enzyme stocks were kept in 50 mM Tris containing 0.1% Tergitol, 50 mM NaCl, and 1 mM $CaCl_2$. TBBL (18) was used from a 0.2 M stock in acetonitrile, and the co-solvent percentage was equalized to 1% in all reaction mixtures. Product formation was monitored spectrophotometrically in 200-μl reaction volumes using 96-well plates by coupling to 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) as described (18). For assays at pH ≤7.0 (below the pK_a of 5,5′-dithiobis(2-nitrobenzoic acid)), 100-μl aliquots taken from 1-ml reactions were transferred at 20-s intervals into 100 μl of buffer containing 5,5′-dithiobis(2-nitrobenzoic acid) and the PON1 inhibitor 2-hydroxyquinoline (2 mM) to quench the enzymatic reaction. The product concentration was subsequently determined by absorbance at 412 nm. Initial velocities were determined by plotting these end point measurements against time (five or more points) and extrapolating a slope for the linear phase. The reported results are the average of at least three independent measurements.

Kinetic Measurements with rePON1 Mutants—The kinetic measurements were performed in buffer containing 50 mM Tris (pH 8.0) and 1 mM $CaCl_2$, and aliphatic lactone hydrolysis was monitored as described previously (6). A range of enzyme concentrations was used depending on the reactivity of the substrate and the mutant. The activities of the mutants and wild type-like rePON1-G2E6 were examined with substrates of PON1 of three subgroups: phosphotriesters (paraoxon, 0.85 mM; and 7-diethylphosphoro-3-cyanocoumarin (DEP-coumarin, 47.5 μM), esters (phenyl acetate, 1 mM; and 2-naphthyl acetate, 0.2 mM), and lactones (dihydrocoumarin, 0.25 mM; δ-valerolactone, 1 mM; and γ-nonalactone, 1 mM). The substrate concentrations were varied according to the solubility, reactivity, and extinction coefficients of each substrate. The reported results are the average of at least two independent measurements. For kinetic parameters determinations, the substrate concentrations were varied in the range of $0.3 \times K_m$ up to $2–3 \times K_m$, except for those cases in which substrate solubility was limiting (phenyl acetate in the case of all mutants, δ-valerolactone in the case of H115Q and the double mutant, and γ-caprolactone in the case of H115Q and H134Q). The percentage of co-solvent (MeOH in case of phenyl acetate and paraoxon, Me_2SO in case of lactones, and acetonitrile in the case of TBBL) was equalized to 1–1.6% in all reactions.

Data Analysis—Kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) were obtained by fitting the data to the Michaelis-Menten equation ($v_0 = k_{cat}[E]_0[S]_0/([S]_0 + K_m)$) using the program KaleidaGraph 5.0. In cases in which solubility limited the initial substrate concentrations, the 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. All data presented are the means ± S.D. of at least three independent experiments. The pH-rate data (k_{cat} ($(k_{cat})^H$) and k_{cat}/K_m ($(k_{cat}/K_m)^H$) values for each pH value) were fitted to a "bell-shaped" model using the equations $(k_{cat})^H = (k_{cat})^{max}/((10^{-pH}/10^{-pK_{a1}}) + (10^{-pK_{a2}}/10^{-pH}) + 1)$ and $(k_{cat}/K_m)^H = (k_{cat}/K_m)^{max}/((10^{-pH}/10^{-pK_{a1}}) + (10^{-pK_{a2}}/10^{-pH}) + 1)$, where $(k_{cat})^{max}$ and $(k_{cat}/K_m)^{max}$ are the plateau values of k_{cat} and k_{cat}/K_m, respectively, and pK_{a1} and pK_{a2} are the apparent pK_a values for the acidic and basic groups, respectively.

Rabbit PON3—Wild-type rabbit PON3 and its mutants were cloned, expressed, and purified analogously to rePON1 variants, except that the elution buffer did not contain glycerol. The expression of PON3 variants was comparatively low (~2 mg/liter of culture) and was entirely dependent on the fusion protein thioredoxin (19). Although SDS-PAGE indicated that the resulting protein was only 30% pure, the kinetic analysis was performed without any further purification because no contaminating lactonase activity was observed. The ratios between the activities of PON1 and PON3 with several aliphatic lactones were similar to those reported by Draganov *et al.* (4) and Billecke *et al.* (20). However, the esterase activities of rabbit PON3 with phenyl acetate and *p*-nitrophenyl acetate were found to be much lower than reported previously (4). The activities of PON3 and its mutants were determined with 5-(thioethyl)butyrolactone (TEBL, 0.22 mM), TBBL (0.27 mM), δ-valerolactone (1 mM), γ-nonalactone (1 mM), and γ-undecanoic lactone (0.5 mM).

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RESULTS

pH-rate Profiles

The hydrolysis of aliphatic lactones is usually measured by the pH indicator assay (5–7), which does not allow wide variations of pH. Thus, the pH-rate profile of rePON1 was determined with TBBL (18), a lactone substrate that is analogous to γ-nonanoic lactone, yet releases, upon hydrolysis of the γ-butyrolactone ring, a thiol moiety that can be detected with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)). An overlay of the pH-rate profile of TBBL with the previously published pH-rate profiles of an ester (2-naphthyl acetate) and a phosphotriester (paraoxon) (12) is provided in Fig. 1. The parameters obtained from the pH-rate profiles are summarized in Table 1.

Overall, the pH-rate profiles of all three substrates are similar, with a fully pronounced acidic shoulder and a minor basic shoulder that is most apparent in the case of 2-naphthyl acetate. With the exception of TBBL, for which K_m values obtained at pH <6.5 were higher than those obtained at pH >6.5, the K_m values did not vary much with pH, as indicated by the similarity of the pH-rate profiles obtained for k_{cat} and k_{cat}/K_m. The difference between the pK_a values obtained for k_{cat} *versus* k_{cat}/K_m can be explained by the fact that the k_{cat}/K_m data provide the pK_a of the free enzyme, whereas the k_{cat} data provide the pK_a of the enzyme-substrate complex. The small differences between the pK_a values obtained with same substrate for k_{cat} *versus* k_{cat}/K_m may therefore reflect a change in the active-site environment upon formation of the enzyme-substrate complex. Notably, the pK_{a1} derived from k_{cat}/K_m is essentially identical for all substrates, thus reflecting the same free enzyme form. The larger differences observed with the pK_{a2} derived from k_{cat}/K_m, especially for paraoxon, may indicate that this substrate might be binding a different conformer of PON1. It should also be noted that pK_{a2} values were largely extrapolated from the data obtained around or even below pK_{a2}; thus, the accuracy of pK_{a2} values is inevitably low.

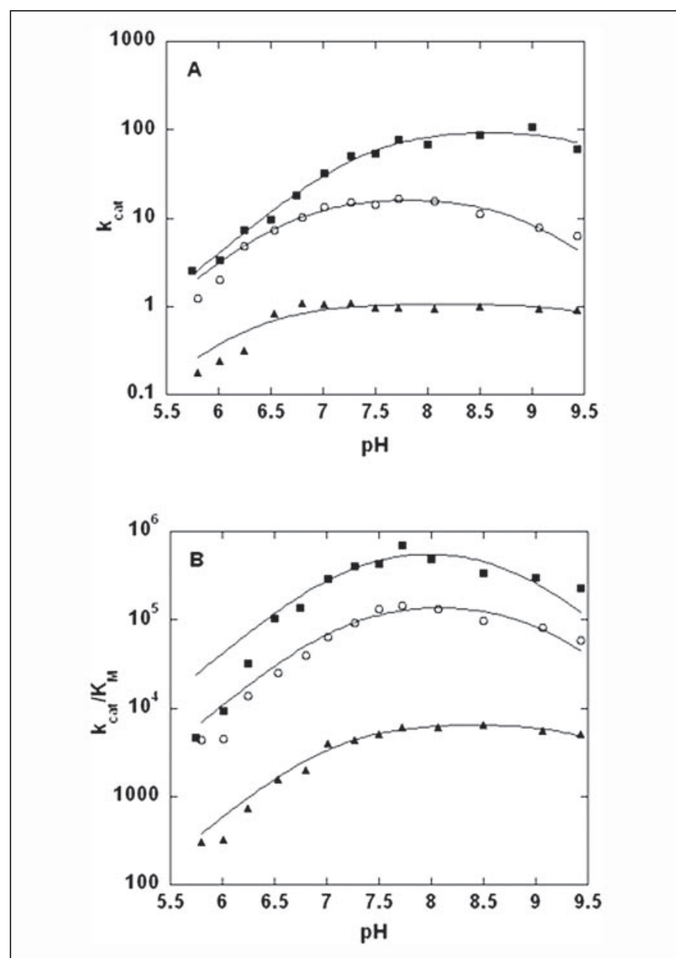

FIGURE 1. **pH-rate profiles of rePON1 with TBBL (■), 2-naphthyl acetate (○), and paraoxon (▲).** Shown are the k_{cat} (*A*) and k_{cat}/K_m (*B*) data. Data were fitted to a bell-shaped model using the equations $(k_{cat})^H = (k_{cat})^{max}/((10^{-pH}/10^{-pK_{a1}}) + (10^{-pK_{a2}}/10^{-pH}) + 1)$ for *A* and $(k_{cat}/K_m)^H = (k_{cat}/K_m)^{max}/((10^{-pH}/10^{-pK_{a1}}) + (10^{-pK_{a2}}/10^{-pH}) + 1)$ for *B*.

As in the case of 2-naphthyl acetate and paraoxon (12), the major acidic shoulder (pK_{a1}) of the lactone substrate may be ascribed to a group that directly participates in catalysis and is active in its basic deprotonated form. The observed pK_a values (6.3–7.4) are most consistent with the imidazole group of histidine, the pK_a of which in aqueous solution is ~6.8 (21). However, because the pK_a values of side chains in proteins and particularly in active sites can vary greatly from their values in solution, other residues could not be excluded on the basis of the pH-rate profiles. The observed pK_a can also correspond to an aspartic or glutamic acid side chain, the pK_a of which is generally ~4, but can be raised in enzyme active sites up to and possibly beyond 6.5 (21). The differences in the acidic pK_a values of the different substrates, specifically those obtained with k_{cat}, may reflect variations in the catalytic mechanism. Most notable is the difference between the pK_{a1} of paraoxon (6.3) and that of TBBL (7.4). Indeed, as shown below, the hydrolysis of paraoxon is not mediated by the same active-site residues that mediate the lactonase activity.

The minor basic shoulder (pK_{a2}) probably reflects a deprotonation of a basic side chain that affects the active site, but is not directly involved in catalysis. Among other possibilities, this basic pK_a might reflect a general deprotonation of lysine side chains (*e.g.* Lys[192]) that causes a mild deactivation of the enzyme.

Active Site of PON1

Although at this stage we cannot completely rule out a nucleophilic mechanism, we have not observed any kinetic indications for the existence of an acyl-enzyme intermediate, not even in substrates with very good leaving groups (6). Thus, we assume the simplest mechanism in which an active-site general base deprotonates a water molecule to generate a hydroxide ion that attacks the phosphoryl/carbonyl of the various substrates. The active site of PON1 (Fig. 2) contains not one but several reasonable candidates for the role of a general base, including four histidines (His[115], His[134], His[285], and His[184]), two aspartates (Asp[183] and Asp[269]), and one glutamic acid (Glu[53]). All these residues are conserved in all mammalian PONs. Asp[269] and Glu[53] participate in the ligation of catalytic Ca^{2+}; and although they are not likely to act as a

TABLE 1
Kinetic parameters extracted from the pH-rate profiles of rePON1

Substrate	$(k_{cat})^{max}$	pK_{a1} (k_{cat} data)	pK_{a2} (k_{cat} data)	$(k_{cat}/K_m)^{max}$	pK_{a1} (k_{cat}/K_m data)	pK_{a2} (k_{cat}/K_m data)
	s^{-1}			$M^{-1} s^{-1}$		
TBBL	104 ± 11	7.4 ± 0.1	~9.8[a]	750,000 ± 150,000	7.2 ± 0.2	8.7 ± 0.2
2-Naphthyl acetate	18 ± 1	6.67 ± 0.09	8.9 ± 0.1	170,000 ± 17,600	7.2 ± 0.1	9.0 ± 0.1
Paraoxon	1.09 ± 0.08	6.3 ± 0.1	~10.0[a]	6980 ± 290	7.04 ± 0.06	~9.8[a]

[a] pK_{a2} values were extrapolated from the data obtained below the actual pK_{a2} and hence can only be estimated.

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general base (let alone as a nucleophile), their titration could, in principle, disrupt the active site and give rise to pK_{a1}. Thus, a mechanism in which Asp^{269} or Glu^{53} is involved in both Ca^{2+} ligation and water deprotonation cannot be totally ruled out at this stage.

His^{115}, being only ~4 Å from the catalytic calcium, is the most obvious candidate for the general base role. As proposed previously (12), it can form a His-His dyad with His^{134}, in which His^{115} is activated by His^{134} via a proton shuttle mechanism. His^{134} itself is far less likely to act directly as a general base because of its distance from the catalytic calcium (8.7 Å). His^{285} is also not far from the catalytic calcium (~7 Å) and was indeed suggested to be involved in catalysis (14). Another possible candidate for the general base role is His^{184}, although its distance from the catalytic calcium is quite long (~11 Å). Asp^{183}, which neighbors His^{184}, could either act itself as a general base or form a dyad with His^{184}, in which His^{184} acts as a general base and Asp^{183} as a proton shuttle. Similar arrangements are observed in other calcium-dependent hydrolases such as secreted phospholipase A_2 and diisopropyl-fluorophosphatase (22, 23). Given this variety of putative catalytic residues, we applied site-directed mutagenesis to identify those side chains that mediate the lactonase activity of mammalian PONs and possibly the other promiscuous activities of PON1.

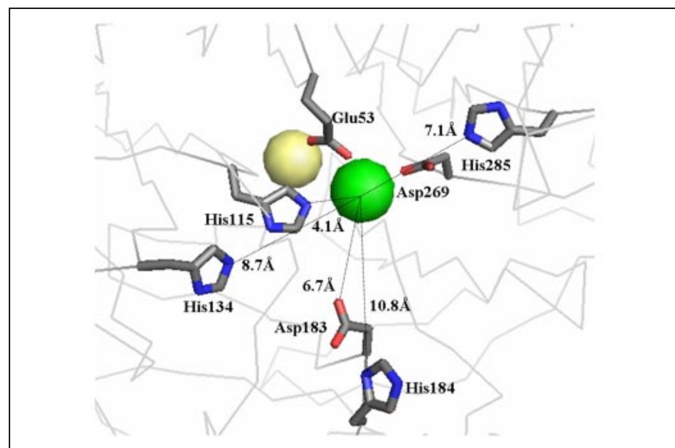

FIGURE 2. **Active site of PON1 (Protein Data Bank code 1V04) (12).** Shown are the structural calcium (*pale yellow*), the catalytic calcium (*green*), and all potentially catalytic residues (Glu^{53} and Asp^{269} (Ca^{2+}-chelating) and Asp^{183}, His^{115}, His^{134}, His^{183}, and His^{285}) and their distances from the catalytic calcium. The images of the active site of rePON1 were created using PyMOL (available at pymol.sourceforge.net/).

Site-directed Mutagenesis and Library Screens

Although an extremely powerful tool, site-directed mutagenesis has its own limitations (24, 25). Mutagenesis in the context of mechanistic studies generally involves substitutions of side chains with side chains that are similar in terms of size and polarity but that are unable to perform the same catalytic function, thus producing a "local" effect. However, even subtle mutations can result in diminished expression, misfolding, or inactivation of the protein ("global" effects). Separating the global effect (arising from a disruption of the structure of the active site or the entire protein) from the local changes that one would actually like to probe is not a trivial matter (26).

The broad range of the hydrolytic activities of PON1 partially solves this problem. Supposing that the different activities are mediated by different active-site residues, there are two possible scenarios. If all activities are reduced by a similar degree, the effect of the mutation is more likely to be global, at least at the level of the active site. If only one type of the activity is affected, one can safely assume that the effect of the mutation is purely local.

To further address the problem of local *versus* global effects, the candidate catalytic residues were mutated to a set of alternative amino acids by generating small libraries that offer multiple substitutions at each position. In the case of His^{115}, His^{184}, and His^{285}, substitutions with all amino acids except proline were explored. The libraries of Asp^{269} and Asp^{183} contained substitutions with all other amino acids.

The libraries were screened for phosphotriesterase, esterase, and lactonase activities using paraoxon, 2-naphthyl acetate, and TBBL, respectively. These screens indicated, on the one hand, whether all substitutions result in an active mutant, suggesting a minor role (if any) for the examined residue, and, on the other hand, which amino acid can replace the examined residue with a minimal effect on the enzyme activity. This library approach obviously provides more information than a single substitution.

The mutants showing the highest activity in library screens (*e.g.* H115A, H115W, H285S, and H184T) were overexpressed and purified. The mutants containing a substitution of histidine with glutamine were created as a default because they are generally accepted as the most conserved mutation. The purified proteins were screened for their activities with substrates of PON1 belonging to four subgroups: ali-

TABLE 2

Activities of selected variants from the mutant library screens (units or μmol of product/min/mg of enzyme) and their percentage relative to wild-type PON1

Data were obtained with purified enzymes; substrate concentrations are provided under "Experimental Procedures." DEP-coumarin, 7-diethylphosphoro-3-cyanocoumarin.

PON1	Phosphotriesters		Dihydrocoumarin	Esters		Lactones	
	Paraoxon	DEP-coumarin		Phenyl acetate	2-Naphthyl acetate	δ-Valerolactone	γ-Nonalactone
Wild-type	1.62 ± 0.06 (100%)	0.450 ± 0.008 (100%)	183 ± 10 (100%)	633 ± 60 (100%)	11.3 ± 0.3 (100%)	142 ± 6 (100%)	28 ± 1 (100%)
H115A	2.70 ± 0.02 (167%)	0.535 ± 0.009 (119%)	670 ± 15 (366%)	1.893 ± 0.006 (0.30%)	0.03 ± 0.01 (0.30%)	4.3 ± 0.1 (3.0%)	0.25 ± 0.04 (0.89%)
H115W	3.2 ± 0.3 (195%)	1.05 ± 0.03 (234%)	477 ± 18 (261%)	0.35 ± 0.02 (0.056%)	0.032 ± 0.006 (0.28%)	0.9 ± 0.2 (0.6%)	0.04 ± 0.02 (0.14%)
H115Q	0.52 ± 0.07 (32%)	0.112 ± 0.008 (25%)	490 ± 24 (268%)	3.67 ± 0.03 (0.58%)	0.116 ± 0.007 (1.0%)	6.9 ± 0.1 (4.9%)	0.33 ± 0.03 (1.2%)
H134Q	9.7 ± 0.3 (602%)	0.171 ± 0.008 (38%)	141 ± 5 (77%)	62.98 ± 0.03 (9.9%)	0.35 ± 0.01 (3.1%)	18 ± 3 (13%)	6.1 ± 0.3 (22%)
H285Q	0.217 ± 0.003 (13%)	0.019 ± 0.001 (4.3%)	41 ± 5 (22%)	74 ± 6 (12%)	2.2 ± 0.1 (19%)	28.0 ± 0.3 (20%)	10.6 ± 0.4 (38%)
H285S	0.86 ± 0.02 (53%)	0.113 ± 0.008 (25%)	23.6 ± 0.9 (13%)	67 ± 2 (10%)	6.1 ± 0.1 (61%)	11.5 ± 0.4 (8.1%)	4.5 ± 0.1 (16%)
H184Q	0.144 ± 0.005 (8.9%)	0.0354 ± 0.0003 (7.9%)	105 ± 5 (57%)	206 ± 8 (32%)	7.4 ± 0.4 (66%)	63 ± 1 (44%)	12.26 ± 0.06 (44%)
H184T	0.151 ± 0.002 (9.3%)	0.0120 ± 0.0002 (2.7%)	31 ± 2 (17%)	170 ± 4 (27%)	6.4 ± 0.3 (57%)	49.26 ± 0.06 (35%)	10.9 ± 0.3 (39%)

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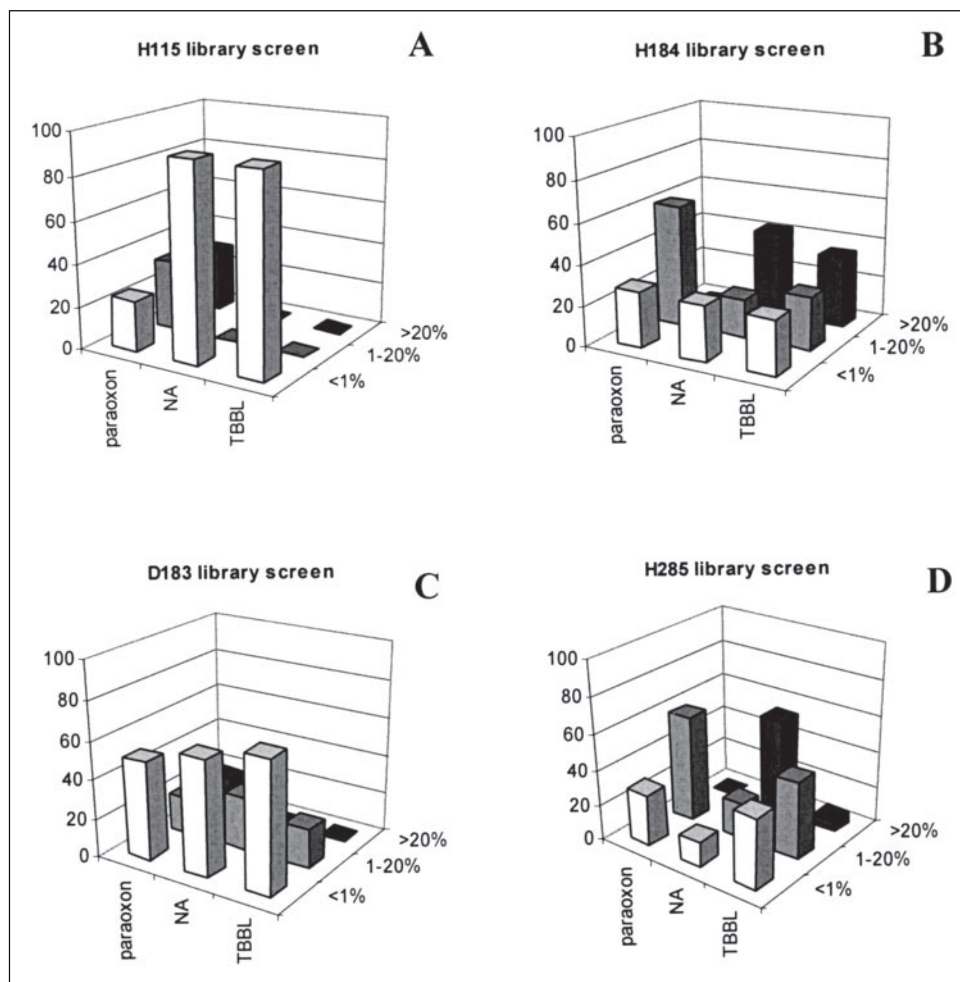

FIGURE 3. **Results of the mutant library screens.** Shown is the number of clones for each activity threshold (<1, 1–20, and >20% of the wild-type activity) obtained in screens of crude lysates of His[115] (*A*), His[184] (*B*), Asp[183] (*C*), and His[285] (*D*) libraries with lactone (TBBL), ester (2-naphthyl acetate), and phosphotriester (paraoxon) substrates.

phatic lactones (δ-valerolactone and γ-nonalactone), phosphotriesters (paraoxon and 7-diethylphosphoro-3-cyanocoumarin), esters (phenyl acetate and 2-naphthyl acetate), and dihydrocoumarin (Table 2).

His[115]—The library screen indicated that all substitutions of His[115] led to a substantial loss (≥99%) of the lactonase and esterase activities (Fig. 3*A*). Indeed, the activities of the purified H115A, H115W, and H115Q mutants with both aryl esters were below 1% relative to wild-type PON1, and their lactonase activities with aliphatic lactones were 0.14–4.9% of those wild-type PON1 (Table 2). However, the hydrolysis of dihydrocoumarin by the His[115] mutants was ~3-fold faster than that by wild-type PON1, indicating that dihydrocoumarin is not a typical lactone substrate of PON1, although it was previously considered as such (3, 12, 20). The phosphotriesterase activity was largely retained in most of the variants of the His[115] library. The most active variants (≤2-fold of the wild-type activity) had substitutions with tryptophan (14), lysine, alanine, and threonine. The results of site-directed mutagenesis of His[115] suggest that different active-site residues mediate the activity of PON1 with the various types of substrates because His[115] appears to participate in lactone and aryl ester hydrolysis, but has little effect on the hydrolysis of phosphotriesters and dihydrocoumarin.

His[134]—Mutagenesis of His[134] to glutamine resulted in the same pattern of activities as mutagenesis of His[115], but the effect of the His[134] mutation was milder (Table 2). The lactonase and esterase activities were 3–22% of those of wild-type PON1, whereas the hydrolysis of dihydrocoumarin and phosphotriesters was barely affected. Thus, our proposed mechanism of ester hydrolysis, based on the His[115]-His[134] dyad (12), is a plausible mechanism for both the lactonase and arylesterase activities of PON1.

His[184]—The His[184] library generally exhibited higher activity with 2-naphthyl acetate and TBBL than with paraoxon (Fig. 3*B*). The activity of all His[184] variants with paraoxon decreased by >5-fold (Fig. 3*B*). In the most active variants, His[184] was replaced with threonine or arginine. Both H184Q and H184T mutants showed a similar pattern: the phosphotriesterase activity decreased to <10% with both of the phosphotriesters tested, whereas other activities were less affected (≤3-fold decrease) (Table 2). Thus, His[184] does not seem to be involved in the lactonase/esterase mechanism, but may participate, if only partially, in phosphotriester hydrolysis.

Asp[183]—The Asp[183] library exhibited more active clones with paraoxon than with naphthyl acetate (Fig. 3*C*), and the activities followed the same pattern, *i.e.* variants that had high activity with paraoxon were active with 2-naphthyl acetate. Active variants had substitutions of Asp[183] with serine, cysteine, and threonine. Thus, Asp[183] is not likely to act as a general base. It does not appear to form a dyad with His[184] either because the library screens of these two residues showed different patterns with paraoxonase and lactonase/esterase activities.

His[285]—The His[285] library contained many clones that were moderately active with all substrates (Fig. 3*D* and Table 2). Notably, the mutation of His[285] to serine (Table 2) caused a mild decrease (1.6–12-fold) in all types of activities, including dihydrocoumarin hydrolysis, which was barely affected by mutations of other residues. The results of His[115] mutagenesis and, to a lesser extent, His[184] mutagenesis showed, how-

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ever, that the phosphotriesterase, lactonase/esterase, and dihydrocoumarin hydrolytic activities are clearly separated in PON1. The facts that His[285] tolerated many substitutions and that these substitutions led to a parallel decrease in activity with all substrates suggest a global effect on the active site, rather than a direct involvement of His[285] in catalysis.

Asp[269]—The Asp[269] library was totally inactive with all substrates examined. This demonstrates that any mutation of Asp[269], which chelates the catalytic calcium, causes a massive disruption of the active site. Another possible scenario is a global effect on the stability and/or expression levels of Asp[269] mutants. Thus, both Asp[269] and Glu[53] play a critical role, presumably in ligating the catalytic calcium, although an additional role as a general base cannot be ruled out completely.

In conclusion, the results of the site-directed mutagenesis experiments suggest a key role for His[115] in lactone and ester hydrolysis. Mutations of all other residues with pK_a values of 6–7 that may act as a general base caused either no or a parallel decrease in all activities of PON1. Thus, we focused our studies on His[115] and its neighboring residue His[134].

Detailed Analysis of the His[115]-His[134] Dyad in PON1 and PON3

The kinetic parameters of the H115Q, H134Q, and H115Q/H134Q mutants were measured with paraoxon, phenyl acetate, and several aliphatic lactones and compared with those of wild-type rePON1 (Table 3). The kinetic parameters of paraoxon hydrolysis did not change much, and the substitution of His[134] with glutamine even increased the k_{cat} of paraoxon hydrolysis so that the catalytic proficiency of the double mutant H115Q/H134Q toward paraoxon remained close to that of wild-type rePON1. However, the catalytic proficiency of phenyl acetate and lactone hydrolysis was significantly affected by the mutation of His[115], the greatest impact being that of k_{cat} decreasing by up to 80-fold. The mutation of His[134] had a smaller effect on the kinetic parameters, and the k_{cat}/K_m values of the His[134] mutant were 5–14-fold lower than those of wild-type rePON1. The catalytic efficiency of the double mutant H115Q/H134Q with lactones and phenyl acetate was ≥100-fold lower than that of wild-type rePON1.

Because the results with PON1 demonstrated the role of His[115] and His[134] in lactone hydrolysis, we wanted to probe the role of these residues in other mammalian PONs. His[115] and His[134] (as well as His[184], His[285], and Asp[183]) are conserved throughout the mammalian PON family. However, the only activity of PON2 is lactonase, and PON3, which is primarily a lactonase, also exhibits traces of paraoxonase activity (k_{cat}/K_m of rabbit PON3 = 0.7 $\text{M}^{-1}\,\text{s}^{-1}$) and weak esterase activity (k_{cat}/K_m for 2-naphthyl acetate hydrolysis = 3.1×10^3 $\text{M}^{-1}\,\text{s}^{-1}$) (19). These differences are also consistent with lactonase being the native activity of all mammalian PONs and with mutations of active-site residues affecting the promiscuous but not the native function (3, 12). The H115Q and H115Q/H134Q mutants of rabbit PON3 were generated, and their lactonase activities were compared with that of wild-type PON3 (Table 4). The activity of the H115Q mutant with lipophilic aliphatic lactones, which have been shown to be the best substrates of PON3, was too low to be determined, and the activities with 5-(thioethyl)butyrolactone (TEBL) and TBBL were significantly reduced. The double mutant H115Q/H134Q exhibited <1% of the wild-type activity with all lactone substrates tested.

DISCUSSION

Non-detrimental Effect of Mutations on PON1 Activities—Interestingly, the activity of PON1 was not totally abolished upon any of the mutations examined, except the mutations of Asp[269] that probably disrupt the binding of the catalytic calcium ion. It is often expected that the activity of an enzyme is essentially abolished upon mutation of a cata-

TABLE 3

Kinetic parameters of rePON1 mutants (percentage relative to wild-type rePON1)

Data were obtained with purified proteins.

rePON1	Paraoxon	Phenyl acetate	δ-Valerolactone	γ-Nonalactone	γ-Caprolactone	TBBL
Wild-type						
k_{cat}, s^{-1}	4.2 ± 0.2	854 ± 105	210 ± 9	31 ± 2	44 ± 1	116 ± 4
K_m, mM	0.86 ± 0.02	1.3 ± 0.2	0.57 ± 0.02	0.39 ± 0.03	1.0 ± 0.1	0.27 ± 0.04
k_{cat}/K_m, $\text{M}^{-1}\,\text{s}^{-1}$	4890 ± 240 (100%)	668,000 ± 40,000 (100%)	370,000 ± 35,000 (100%)	78,000 ± 1600 (100%)	43,000 ± 6700 (100%)	440,000 ± 55,000 (100%)
H115Q						
k_{cat}, s^{-1}	1.00 ± 0.09 (24%)	10.5 ± 0.5 (1.2%)	69.4 ± 5.6 (33%)	0.38 ± 0.05 (1.2%)	2.27 ± 0.17 (5.2%)	7.6 ± 0.3 (6.5%)
K_m, mM	1.35 ± 0.16 (157%)	8.1 ± 0.9 (623%)	8.5 ± 1.0 (1490%)	1.54 ± 0.35 (395%)	3.8 ± 0.5 (380%)	1.6 ± 0.1 (596%)
k_{cat}/K_m, $\text{M}^{-1}\,\text{s}^{-1}$	738 ± 15 (15%)	1310 ± 87 (0.2%)	8200 ± 380 (2.2%)	250 ± 240 (0.3%)	600 ± 40 (1.4%)	4720 ± 168 (1.1%)
H134Q						
k_{cat}, s^{-1}	12.7 ± 0.6 (302%)	185 ± 7 (22%)	40.0 ± 1.4 (19%)	5.57 ± 0.24 (18%)	25.0 ± 1.2 (57%)	19.4 ± 0.2 (17%)
K_m, mM	0.75 ± 0.05 (87%)	3.6 ± 0.1 (277%)	0.95 ± 0.08 (167%)	0.89 ± 0.05 (228%)	2.97 ± 0.07 (297%)	0.68 ± 0.03 (252%)
k_{cat}/K_m, $\text{M}^{-1}\,\text{s}^{-1}$	17,000 ± 230 (348%)	50,900 ± 494 (7.6%)	42,200 ± 1900 (11%)	6240 ± 104 (8.0%)	8400 ± 230 (19%)	28,600 ± 1500 (6.5%)
H115Q/H134Q						
k_{cat}, s^{-1}	4.7 ± 0.1 (112%)	7.4 ± 0.8 (0.9%)	ND[a]	ND[b]	ND[b]	1.74 ± 0.05 (1.5%)
K_m, mM	1.18 ± 0.07 (137%)	9.7 ± 1.6 (746%)	>10 (>1750%)			1.83 ± 0.09 (678%)
k_{cat}/K_m, $\text{M}^{-1}\,\text{s}^{-1}$	3980 ± 180 (81%)	773 ± 37 (0.1%)	2970 ± 174 (0.8%)			965 ± 1 (0.2%)

[a] k_{cat} could not be determined because of the large K_m (no saturation of the Michaelis-Menten curve).

[b] No activity was above the detection threshold ($k_{cat}/K_m \sim 200$ $\text{M}^{-1}\,\text{s}^{-1}$).

TABLE 4

Activities of PON3 mutants (units or μmol of product/min/mg of enzyme) and their percentage relative to wild-type PON3

Data were obtained with enzymes purified to ~30% homogeneity (see "Experimental Procedures"). TEBL, 5-(thioethyl)butyrolactone.

Substrate	PON3		
	Wild-type	H115Q	H115Q/H134Q
TEBL	1.91 ± 0.08 (100%)	0.020 ± 0.003 (1.1%)	0.003 ± 0.002 (0.2%)
TBBL	8.2 ± 0.2 (100%)	0.345 ± 0.005 (4.2%)	0.050 ± 0.005 (0.6%)
δ-Valerolactone	0.72 ± 0.03 (100%)	ND[a] (<1%)	ND (<1%)
γ-Nonalactone	7.2 ± 0.1 (100%)	ND (<1%)	ND (<1%)
γ-Undecanoic lactone	8.2 ± 0.2 (100%)	ND (<1%)	ND (<1%)

[a] Background level activity (<1% of wild-type PON3).

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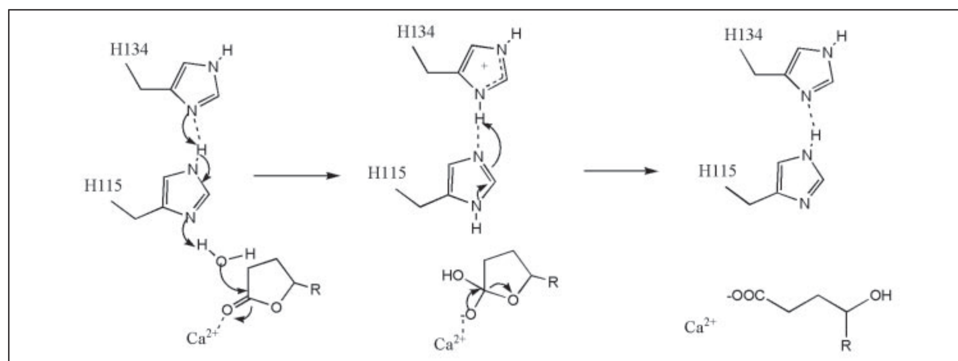

FIGURE 4. **Proposed lactonase mechanism of PON1.**

lytic residue that mediates it. However, this is not so with PON1 or with many other enzymes (24). The retention of considerable activity after mutation of residues that play a central role in catalysis can be ascribed to the substantial plasticity of active sites and the catalytic mechanism. Thus, the removal of a key catalytic residue may significantly alter the mechanism, but not abolish the enzyme activity (24). As mentioned above, the active site of PON1 contains a calcium ion that stabilizes the negatively charged transition states and intermediates formed during hydrolysis of all substrate types and several other residues that may serve as a general base. It seems that, for every type of a substrate, there is a "preferred" histidine or possibly another side chain that can serve as a general base for that substrate type. Upon mutation of one of these residues, the activity decreases substantially, but is not completely abolished, partly because other residues may act as a general base. It also seems that the promiscuous activities of PON1, such as hydrolysis of dihydrocoumarin or paraoxon, could be mediated by several alternative residues (*e.g.* His[115] or His[184]) and therefore show even higher plasticity than the lactonase activity. Moreover, even in the absence of alternative general bases in the active site, there are cases in which mutants of a catalytic His residue retain significant activity because of a change in the catalytic mechanism. For example, the H48Q mutant of phospholipase A_2 exhibits significant activity (2.8% of the wild-type activity) presumably because of the ability of Gln to act as a general base (27). There are many other cases in which mutations of key catalytic residues do not abolish activity (24). This demonstrates that enzyme active sites combine many elements and that their activities rarely rely on one or even a few residues. Other parameters such as effective binding and optimal alignment of the substrate in the active site, mediated in the case of PONs by the calcium ion, lead to the stabilization of the transition state and can result in considerable rate accelerations in the absence of a general base. Thus, the fact that mutating His[115] does not completely abolish activity does not contradict our proposed mechanism in which His[115] plays a key role in the activation of water molecules and in the generation of the attacking hydroxide.

The His[115]-His[134] Dyad Mediates the Lactonase Activity of PONs—On the basis of the pH-rate profiles of TBBL, naphthyl acetate, and paraoxon, we found that all hydrolytic activities of PON1 (lactonase, esterase, and phosphotriesterase) are mediated by an amino residue(s) with a pK_a of 6.3–7.4 that is active in the deprotonated form. Site-directed mutagenesis studies demonstrated that, although all these activities take place in the same active site (6), the residues that mediate the lactonase and esterase activities are different from those involved in the phosphotriesterase activity.

The results presented here indicate that the hydrolysis of lactones and esters is mediated by the His[115]-His[134] dyad (Fig. 4). We propose a mechanism in which His[115] acts as a general base, deprotonates a water molecule, and generates a hydroxide ion that attacks the carbonyl of the lactone/ester substrate. His[134] activates His[115] by serving as a proton shuttle. Catalytic Ca^{2+} serves as an oxyanion hole and stabilizes the negative intermediate produced by the attacking hydroxide ion. The mutagenesis results support the dyad mechanism and an auxiliary role for His[134]. The impact of the His[134] mutation in the double mutant H115Q/H134Q is smaller than expected by assuming simple additivity of the mutations (28). This is most clearly observed in the case of phenyl acetate: the H115Q mutation caused a 510-fold decrease in the k_{cat}/K_m for phenyl acetate hydrolysis, and the H134Q mutation caused another ~13-fold decrease. However, the k_{cat}/K_m of the double mutant decreased by ~860-fold, instead of the ~6700-fold decrease expected if these mutations would be simply additive. Similarly, the H115Q mutation caused an ~45-fold decrease in the k_{cat}/K_m for δ-valerolactone, and the H134Q mutation caused another 9-fold decrease. The k_{cat}/K_m of the double mutant was, however, only 2.8-fold less active than that of the H115Q mutant.

The fact that the PON3 lactonase activity, which is the only apparent activity of this enzyme, was affected by mutations of His[115] and His[134] similarly to PON1 demonstrates that the mechanism based on the His[115]-His[134] dyad is probably common to all mammalian PONs. PON1, PON2, and PON3 share 60–70% sequence identity, and the only activity common to all of them is the lactonase activity (4). Thus, these enzymes are lactonases that share the same mechanism of catalysis, but have different substrate selectivity and different patterns of promiscuity.

The accompanying article (16) describes the effect of the His[115] and His[134] mutations on the anti-atherogenic properties of PON1. The H115Q and H134Q mutants of PON1 were found to bind high density lipoprotein. However, their ability to inhibit low density lipoprotein oxidation was lower compared with wild-type PON1, and the double mutant H115Q/H134Q had no effect at all. The stimulation of macrophage cholesterol efflux was also markedly reduced by mutations of His[115] and His[134]. In all these cases, the His[115] mutation had a larger effect compared with the His[134] mutation, and the double mutant exhibited no detectable activity. These results demonstrate that the anti-atherogenic properties of PON1 are also mediated by the His[115]-His[134] dyad and that these properties are directly related to the lactonase function of PON1.

Histidine often serves as a general base in hydrolytic enzymes by subtracting a proton from a water molecule to produce a hydroxide ion that attacks the carbonyl/phosphoryl of the substrate or of an acyl-enzyme intermediate (*e.g.* in serine hydrolases). However, histidine dyads are among the least common active-site arrangements known (29). Histidine typically forms a dyad with aspartic or glutamic acid, which increases the basicity of the imidazole ring by a proton shuttle mechanism (29). For example, in phospholipase A_2, the water is deprotonated by His[48], which is activated by Asp[99] (23). The active site of diisopropyl-fluorophosphatase, which shares the same fold (six-bladed β-propeller) with PON1 and also has two calcium ions in the central

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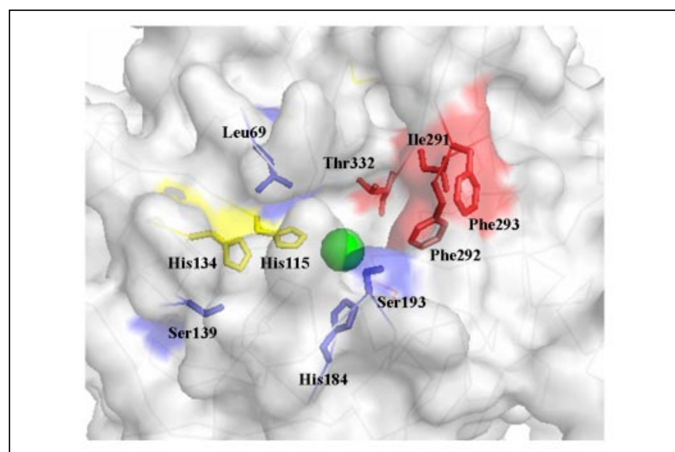

FIGURE 5. **Surface view of the PON1 active site (Protein Data Bank code 1V04) (12).** Shown are the catalytic His[115]-His[134] dyad (*yellow*), His[184], and the residues that govern the substrate selectivity of the lactonase/esterase activities (*red*) and the phosphotriesterase activity (*blue*).

tunnel, contains a His-Glu dyad in which His[287] serves as a general base, and Glu[37] increases its basicity (30). A His-His dyad was previously assigned in the active site of the phosphotransferase domain of glucose permease, in which His[83] acts as a nucleophile and His[68] as an auxiliary (29, 31). Here, we have described another His-His dyad, the geometry of which is very similar to that of His[83]-His[68] in glucose permease, in which His[115] acts as a general base, and His[134] increases its basicity.

Promiscuity of PON1—Despite extensive studies, the catalytic residue(s) responsible for the promiscuous phosphotriesterase activity of PON1 remain largely unclear. Nevertheless, this study clearly indicates that this activity and other activities such as the hydrolysis of dihydrocoumarin are mediated by residues other than those that mediate the lactonase activity.

The mutation of His[115], which is important for hydrolysis of lactones and esters (14, 15), did not have much effect on the phosphotriesterase activity of PON1. The mutations of His[184] caused a >10-fold decrease in this activity, but there is no other evidence that ascribes a role for His[184] in catalysis. A substantial decrease in the phosphotriesterase activity (>130-fold) was obtained only when all three histidines (His[115], His[134], and His[184]) were mutated to glutamine (data not shown). However, because the esterase and lactonase activities were totally abolished in the triple mutant and because the hydrolysis of dihydrocoumarin decreased to <5% of the wild-type level, the dramatic reduction in the phosphotriesterase activity could be the result of a global effect of three parallel mutations. Thus, at this stage, we could not identify a single residue that acts as the general base with the promiscuous phosphotriester substrates. It could also be that the hydrolysis of phosphotriesters (low catalytic activity, $k_{cat}/K_m < 10^4\ \text{M}^{-1}\ \text{s}^{-1}$) and the hydrolysis of dihydrocoumarin (a highly activated substrate) are accomplished primarily by transition state stabilization through the calcium ion and an attack by an active-site water molecule.

PON1 Active-site Architecture—At present, there are no structures of PON1 in complex with a substrate analog or inhibitor. However, data derived from this study and from our directed evolution studies aimed at increasing several PON1 activities provide some insights as to the active-site architecture and mode of substrate binding. The residues that were found to be mutated in the course of directed evolution experiments and to affect the lactonase/esterase or phosphotriesterase activity appear to be located in different areas of the active site (Fig. 5). It can be seen that the putative catalytic dyad His[115]-His[134] is located at the opposite side of a hydrophobic cluster composed of Ile[291], Phe[292], and Phe[293] at the upper part of the active-site wall and Thr[332] located at the lower part. Many mutations of these four residues that affect the esterase and lactonase activities have been found (3, 12). A likely arrangement is therefore that lactones are located in the active site so that the carbonyl moiety points toward the calcium ion at the bottom of the cavity; the alkoxide (or phenoxide in the case of aryl esters) faces the His dyad; and the "back" of the lactone ring and other hydrophobic parts of the substrate face the opposite side marked by residues 332 and 291–293. The residues that affect the phosphotriesterase activity of PON1 (Leu[69], Ser[139], and Ser[193]) are generally in other regions of the active site (Fig. 5), and so is His[184], which may take part in hydrolysis of phosphotriesters. The location of the above residues and the results presented here are all consistent with the mode of phosphotriester binding being significantly different from that of lactone binding.

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