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\(^{260}\)). 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.

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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Esterase activities of PON1 are mediated by the His\textsuperscript{115}–His\textsuperscript{134} dyad and rule out other active-site residues, including His\textsuperscript{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 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, Fluuka, 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 blunted-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 a 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 Gln replacement libraries were generated by replacing His codons with DNS codons encoding all amino acids except Asp, 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) activity.

Expression and Purification of rePON1-G2E6 Mutants—Wild type-like rePON1-G2E6 and its various mutants were expressed as fusion proteins with thioredoxin and His6 tags and purified as described previously (19), except that mutant proteins were eluted from a nickel-chelating lactonase activity was observed. The ratios between the activities of rePON1-G2E6 and its various mutants were examined with substrates of PON1 of three subgroups: phosphotriesters (paraoxon, 0.85 mM; and 7-diethylphosphorono-3-cyanocoumarin (DEP-coumarin, 47.5 μM), esters (phenyl acetate, 1 mM; and 2-naphthyl acetate, 0.2 mM), and lactones (2-hydroxycoumarin, 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 parameter determinations, the substrate concentrations were varied in the range of 0.3 × Km up to 2–3 × Km, 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, MeSO in case of lactones, and acetone in the case of TBBL) was equalized to 1–1.6% in all reactions.

Data Analysis—Kinetic parameters (kcat, Km) were obtained by fitting the data to the Michaelis-Menten equation (v0 = kcat[E]0[S]0/([S]0 + Km)) 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 (v0 = [S]0kcat/Km + kcat/Km, where kcat/Km was deduced from the slope. All data presented are the means ± S.D. of at least three independent experiments. The pH-rate data (kcat/ Km) and kcat/Km values for each pH value were fitted to a "bell-shaped" model using the equations (kcat/ Km) = (kcat/Km)\textsuperscript{max}/(10^(-pH/K0) + 10^(-pH/2K0)) + 1) and (kcat/ Km)\textsuperscript{max}/(10^(-pH/K0) + 10^(-pH/2K0)) + 1), where (kcat/Km)\textsuperscript{max} and (kcat/Km)\textsuperscript{max} are the plateau values of kcat and kcat/Km, respectively, and pK\textsubscript{a} and pK\textsubscript{a} are the apparent pK\textsubscript{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).
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 thiole 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 $K_a$ 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 $pK_a$ 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 $pK_a$ 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 $pK_a$ values were largely extrapolated from the data obtained around or even below $pK_{a1}$; thus, the accuracy of $pK_a$ values is inevitably low.

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 paraoxon, 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. Lys137) 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 only but several reasonable candidates for the role of a general base, including four histidines (His115, His133, His184, and His185), two aspartates (Asp183 and Asp260), and one glutamic acid (Glu53). All these residues are conserved in all mammalian PONs. Asp260 and Glu53 participate in the ligation of catalytic Ca$^{2+}$; and although they are not likely to act as a

![FIGURE 1. pH-rate profiles of rePON1 with TBBL (II), 2-naphthyl acetate (C), and paraoxon (A). Shown are the $k_{cat}$ (A) and $k_{cat}/K_m$ (B) data. Data were fitted to a bell-shaped model using the equations $k_{cat}^x = (k_{cat}^{max})^x/((10^{-pH})^{10^{-pH}}) + 10^{-pH} + 1$ for A and $k_{cat}/K_m^x = (k_{cat}/K_m^{max})^x/((10^{-pH})^{10^{-pH}}) + 10^{-pH} + 1$ for B.]

**TABLE 1**

| Substrate       | $(k_{cat})^{max}$ | $pK_{a1}$ (data) | $pK_{a2}$ (data) | $(k_{cat}/K_m)^{max}$ | $pK_{a1}$ ($k_{cat}/K_m$ data) | $pK_{a2}$ ($k_{cat}/K_m$ data) |
|-----------------|-------------------|-----------------|-----------------|----------------------|-------------------------------|-------------------------------|
| TBBL            | 104 ± 11          | 7.4 ± 0.1       | −9.8a           | 750,000 ± 150,000    | 7.2 ± 0.2                     | 8.7 ± 0.2                     |
| 2-Naphthyl acetate | 18 ± 11          | 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       | −10.0a          | 6980 ± 290           | 7.04 ± 0.06                   | −9.8a                         |

*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 $pK_a$. 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 $\sim 4 \text{ Å}$ 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$^{115}$, 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 ($\sim 7 \text{ Å}$). His$^{269}$ is also not far from the catalytic calcium ($\sim 7 \text{ Å}$) and was indeed suggested to be involved in catalysis (14). Another possible candidate for the general base role is His$^{184}$, 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.

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$^{269}$, 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 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 conservative mutation. The purified proteins were screened for their activities with substrates of PON1 belonging to four subgroups: ali-
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).

His115 — The library screen indicated that all substitutions of His115 led to a substantial loss (≥99%) of the lactonase and esterase activities (Fig. 3A). 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 His115 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 His115 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 His115 suggest that different active-site residues mediate the activity of PON1 with the various types of substrates because His115 appears to participate in lactone and aryl ester hydrolysis, but has little effect on the hydrolysis of phosphotriesters and dihydrocoumarin.

His134 — Mutagenesis of His134 to glutamine resulted in the same pattern of activities as mutagenesis of His115, but the effect of the His134 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 His115-His134 dyad (12), is a plausible mechanism for both the lactonase and arylesterase activities of PON1.

His184 — The His184 library generally exhibited higher activity with 2-naphthyl acetate and TBBL than with paraoxon (Fig. 3B). The activity of all His184 variants with paraoxon decreased by >5-fold (Fig. 3B). In the most active variants, His184 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, His184 does not seem to be involved in the lactonase/esterase mechanism, but may participate, if only partially, in phosphotriester hydrolysis.

Asp183 — The Asp183 library exhibited more active clones with paraoxon than with naphthyl acetate (Fig. 3C), 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 Asp183 with serine, cysteine, and threonine. Thus, Asp183 is not likely to act as a general base. It does not appear to form a dyad with His184 either because the library screens of these two residues showed different patterns with paraoxonase and lactonase/esterase activities.

His285 — The His285 library contained many clones that were moderately active with all substrates (Fig. 3D and Table 2). Notably, the mutation of His285 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 His115 mutagenesis and, to a lesser extent, His184 mutagenesis showed, how-
ever, that the phosphotriesterase, lactonase/esterase, and dihydrocoumarin hydrolytic activities are clearly separated in PON1. The facts that His285 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 His285 in catalysis.

Asp269. The Asp269 library was totally inactive with all substrates examined. This demonstrates that any mutation of Asp269, 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 Asp269 mutants. Thus, both Asp269 and Glu53 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 His115 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 His115 and its neighboring residue His134.

Detailed Analysis of the His115-His134 Dyad in PON1 and PON3

The kinetic parameters of the H115Q and 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 His134 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 His115, the greatest impact being that of $k_{cat}$, decreasing by up to 80-fold. The mutation of His134 had a smaller effect on the kinetic parameters, and the $k_{cat}/K_m$ values of the His115 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 $\geq$100-fold lower than that of wild-type rePON1.

Because the results with PON1 demonstrated the role of His115 and His134 in lactone hydrolysis, we wanted to probe the role of these residues in other mammalian PONs. His115 and His134 (as well as His184, His285, and Asp183) 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 M$^{-1}$ s$^{-1}$) and weak esterase activity ($k_{cat}/K_m$ for 2-naphthyl acetate hydrolysis = $3.1 \times 10^3$ M$^{-1}$ 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-(thioethy1)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 Asp269 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)**

| rePON1 | Paraoxon | Phenyl acetate | $\delta$-Valerolactone | $\gamma$-Nonalactone | $\gamma$-Caprolactone | TBBL |
|--------|----------|----------------|-----------------------|----------------------|-----------------------|------|
| Wild-type | $k_{cat}$ s$^{-1}$ | 4.2 ± 0.2 | 854 ± 105 | 210 ± 9 | 31 ± 2 | 44 ± 1 | 116 ± 4 |
| | $K_m$ M | 0.56 ± 0.02 | 1.3 ± 0.2 | 0.57 ± 0.02 | 0.39 ± 0.03 | 1.0 ± 0.1 | 0.27 ± 0.04 |
| | $k_{cat}/K_m$ M$^{-1}$ s$^{-1}$ | 4890 ± 200 (100%) | 668,000 ± 40,000 (100%) | 370,000 ± 35,000 (100%) | 78,000 ± 160 (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%) |
| | $K_m$ M | 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%) |
| | $k_{cat}/K_m$ M$^{-1}$ 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$^{-1}$ | 1.27 ± 0.63 (302%) | 185 ± 7 (22%) | 40.0 ± 14 (19%) | 5.57 ± 0.24 (18%) | 25.0 ± 1.2 (57%) | 19.4 ± 0.2 (17%) |
| | $K_m$ M | 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%) |
| | $k_{cat}/K_m$ M$^{-1}$ 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* | ND* | ND* | 1.74 ± 0.05 (1.5%) |
| | $K_m$ M | 1.18 ± 0.07 (137%) | 9.7 ± 1.6 (746%) | >10 (>1750%) | 1.83 ± 0.09 (678%) | 965 ± 1 (0.2%) |
| | $k_{cat}/K_m$ M$^{-1}$ s$^{-1}$ | 3980 ± 180 (81%) | 773 ± 37 (0.1%) | 2970 ± 174 (0.8%) | 3980 ± 180 (81%) | 773 ± 37 (0.1%) | 2970 ± 174 (0.8%) |

* $k_{cat}$ could not be determined because of the large $K_m$ (no saturation of the Michaelis-Menten curve).
* No activity was above the detection threshold ($k_{cat}/K_m$ $\geq$ 200 M$^{-1}$ s$^{-1}$).

### TABLE 4

**Activities of PON3 mutants (units or $\mu$mol 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 |
|-----------|------|
| TEBL | 1.91 ± 0.08 (100%) | 0.020 ± 0.003 (1.1%) |
| TBBL | 8.2 ± 0.2 (100%) | 0.345 ± 0.005 (4.2%) |
| $\delta$-Valerolactone | 0.72 ± 0.03 (100%) | ND (<1%) |
| $\gamma$-Nonalactone | 7.2 ± 0.1 (100%) | ND (<1%) |
| $\gamma$-Undecanonic lactone | 8.2 ± 0.2 (100%) | ND (<1%) |

* Background level activity (<1% of wild-type PON3).
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. His115 or His184) 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 A2 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 His115 does not completely abolish activity does not contradict our proposed mechanism in which His115 plays a key role in the activation of water molecules and in the generation of the attacking hydroxide.

The His115-His134 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 His115-His134 dyad (Fig. 4). We propose a mechanism in which His115 acts as a general base, deprotonates a water molecule, and generates a hydroxide ion that attacks the carbonyl of the lactone/ester substrate. His134 activates His115 by serving as a proton shuttle. Catalytic Ca^{2+} serves as an oxynion hole and stabilizes the negative intermediate produced by the attacking hydroxide ion. The mutagenesis results support the dyad mechanism and an auxiliary role for His134. The impact of the His134 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 β-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 His115 and His134 similarly to PON1 demonstrates that the mechanism based on the His115-His134 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 His115 and His134 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 His115 and His134. In all these cases, the His115 mutation had a larger effect compared with the His134 mutation, and the double mutant exhibited no detectable activity. These results demonstrate that the anti-atherogenic properties of PON1 are also mediated by the His115-His134 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 hydrolyases). 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 His46, which is activated by Asp99 (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
tunnel, contains a His-Glu dyad in which His115 serves as a general base, and Glu294 increases its basicity (30). A His-Glu dyad was previously assigned in the active site of the phosphotransferase domain of glucose permease, in which His115 acts as a nucleophile and His134 as an auxiliary (29, 31). Here, we have described another His-Glu dyad, the geometry of which is very similar to that of His83-His68 in glucose permease, in which His115 acts as a general base, and His134 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 His115, 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 His134 caused a >10-fold decrease in this activity, but there is no other evidence that ascribes a role for His134 in catalysis. A substantial decrease in the phosphotriesterase activity (>30-fold) was obtained only when all three histidines (His115, His134, and His184) 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 His115-His134 is located at the opposite side of a hydrophobic cluster composed of Ile291, Phe292, and Phe293 at the upper part of the active-site wall and Thr132 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 329 and 291–293. The residues that affect the phosphotriesterase activity of PON1 (Leu69, Ser130, and Ser193) are generally in other regions of the active site (Fig. 5), and so is His184, 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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