# Identification of Residues Essential for Human Paraoxonase (PON1) Arylesterase/ Organophosphatase Activities†

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ABSTRACT: Human serum paraoxonase (PON1) is a calcium-dependent organophosphatase. To identify residues essential for PON1 activity, we adopted complementary approaches based on chemical modification and site-directed mutagenesis. To detect $^{45}Ca^{2+}$ binding to native and chemically modified PON1, we performed nondenaturating gel electrophoresis. The environment of calcium-binding sites was probed using the $Ca^{2+}$ analogue, terbium. $Tb^{3+}$ binds to calcium-binding sites as shown by displacement of $^{45}Ca^{2+}$ by $Tb^{3+}$. Binding of $Tb^{3+}$ is accompanied by a complete loss of enzyme activity. PON1 chemical modification with the Trp-selective reagent, *N*-bromosuccinimide, and the Asp/Glu-selective, dicyclohexylcarbodiimide, established that Trp and Asp/Glu residues are components of the PON1 active center and calcium-binding sites. Additional evidence for the presence of a Trp residue in the PON1 calcium-binding sites was a characteristic fluorescence emission at 545 nm from the PON1−$Tb^{3+}$ complex and abolishment of that fluorescence upon modification by *N*-bromosuccinimide. The importance of aromatic/hydrophobic character of the residue 280 was demonstrated by site-directed mutagenesis: the W280F mutant was fully active while the W280A and W280L mutants had markedly reduced activity. Twelve amino acids among conserved His and Asp/Glu residues were found essential for PON1 arylesterase and organophosphatase activities: H114, H133, H154, H242, H284, D53, D168, D182, D268, D278, E52, and E194. Finally, the cysteines constituting the PON1 disulfide bond (C41 and C352) were essential, but the glycan chains linked to Asn 252 and 323 were not essential for PON1 secretion and activity.

In the past fifteen years, the search for organophosphate hydrolases (OPases[1]) to be used as therapeutic or decontaminating agents against organophosphate (OP) poisoning has been extensively developed. Several candidates have been considered: bacterial OPases, including the phosphotriesterase (PTE, EC 3.1.8.1.) from *Pseudomonas diminuta* (*1*−*3*) and the halophilic prolidase (EC 3.4.13.9.) from the halotolerant *Alteromonas* (*4*); and eukaryotic OPases, mostly butyrylcholinesterase (BuChE, EC 3.1.1.8.) engineered to hydrolyze the nerve agents sarin, soman, and VX (*5*), DFPase isolated from squid giant axon (*6*) and human serum paraoxonase (PON1; EC 3.1.8.1.) (*7*, *8*).

Table 1: Catalytic Efficiency $k_{cat}/K_m$ ($M^{-1}$ $min^{-1}$) of Human and Bacterial Organophosphate Hydrolases

| | human PON1[a] | | *Pseudomonas diminuta* |
|---|---|---|---|
| OP | A (Q191) | B (R191) | PTE |
| Paraoxon | $6.8 \times 10^5$ [b] | $2.4 \times 106$ [b] | $2.0 \times 10^9$ [c] |
| DFP | $3.7 \times 10^4$ [d] | nd | $5.8 \times 10^8$ [e] |
| Sarin | $9.1 \times 10^5$ [f] | $6.8 \times 104$ [f] | $4.8 \times 10^6$ [g] |
| Soman | $2.8 \times 10^6$ [f] | $2.1 \times 106$ [f] | $6.0 \times 10^5$ [g] |
| VX | nd | nd | $4.1 \times 10^4$ [h] |

[a] A (Gln at position 191) and B (Arg at position 191) phenotype as defined in ref *9*. nd, not determined. [b] pH 7.4 at 37 °C (*10*). [c] pH 9.0 at 25 °C (*11*). [d] pH 7.4 at 25 °C (D. Josse, unpublished work). [e] pH 7.2 at 37 °C (*2*). [f] pH 8.5 at 25 °C (*12*, *13*). [g] pH 7.2 at 37 °C (*14*). [h] pH 8.0 at 28 °C (*15*).

Human PON1 is interesting as a potential therapeutic agent against nerve agent poisoning for at least three reasons. First, its catalytic efficiency against sarin and soman is similar to that of PTE from *Pseudomonas diminuta* (Table 1). These two enzymes and the prolidase from *Alteromonas* are the most active OPases known against these nerve agents. Second, its role as a therapeutic countermeasure against OP

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[1] Abbreviations: OPase, organophosphate hydrolase; OP, organophosphate; PTE, phosphotriesterase; DFP, diisopropylfluorophosphate; BuChE, butyrylcholinesterase; PON, paraoxonase; PCR, polymerase chain reaction; MES, (2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NBS, *N*-bromosuccinimide; HNB, 2-hydroxy-5-nitrobenzyl bromide; MNB, 2-methoxy-5-nitrobenzyl bromide; DMSB, dimethyl sulfonium bromide; DCC, dicyclohexylcarbodiimide; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; WK, Woodward's reagent K; DEPC, diethylpyrocarbonate; NEM, *N*-ethylmaleimide; DTNB, dithiobisnitrobenzoate; PCMB, *p*-chloromercuribenzoate; DNFB, dinitrofluorobenzene; TNBS, trinitrobenzenesulfonic acid; NAI, *N*-acetylimidazole; NBI, *N*-butyrylimidazole; DEAE, diethylaminoethyl; SEAP, secreted alkaline phosphatase; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris buffer saline; VX, *O*-ethyl *S*-(diisopropylaminoethyl) methylphosphonothiolate.

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poisoning was recently demonstrated in PON1 knockout mice (*16*). Third, this is a naturally occurring OPase in human plasma that could be used in multiinjection therapeutic protocols without causing immunological responses. Our long-term goal is to design engineered mutants for human PON1 that could be used in the prophylaxy and treatment of OP intoxication. Our first step to this aim was to identify the active site components of PON1.

PON1 is a 354 amino acid enzyme that shows no similarity to proteins outside of the PON family (*17*), and whose 3D structure has not been determined. It has three cysteine residues in positions 41, 283, and 352; C41 and C352 form a disulfide bond while C283 is free (*18*). Two glycan chains are linked to asparagine residues in positions 252 and 323 (*19*). PON1 is a high-density lipoprotein-associated enzyme possessing a broad substrate specificity mostly toward OPs and arylesters (*20*). No physiological function has yet been assigned to PON1. However, a physiological function of PON1 could be related to lipoprotein metabolism but physiological substrates have not yet been clearly identified (*21*). The catalytic activity of PON1 is strictly dependent on the presence of calcium ions. According to Kuo and La Du (*22*), two calcium ions are bound to PON1: one is bound in the catalytic site ($K_d = 6.6 \times 10^{-6}$ M), and the second has a structural role ($K_d = 3.6 \times 10^{-7}$ M).

Two PON1-like genes have been identified and designated PON2 and PON3 (*17*, *23*). Deduced amino acid sequences of human PON2 and PON3 exhibit about 60% identity to the amino acid sequence of human PON1. Since isolation of human PON2 and PON3 has not yet been possible, it has not been established whether these proteins have arylesterase and OPase activities.

Two complementary approaches were adopted to identify essential amino acid residues of PON1: group-selective labeling and site-directed mutagenesis. The first approach was used to determine amino acid residues potentially important for PON1 activity. The second approach allowed us to identify residues that are essential for PON1 activity among conserved amino acids belonging to the group of selectively labeled residues. Our next aim was to identify residues essential to the PON1 calcium-binding sites. Finally, the importance of the disulfide bond and glycosylation for expression, secretion, and activity of PON1 was investigated by site-directed mutagenesis.

## MATERIALS AND METHODS

*Chemicals.* The suppliers for the following chemicals were Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), or Fluka (Ronkonkoma, NY): *N*-bromosuccinimide (NBS), 2-hydroxy-5-nitrobenzyl bromide (HNB), 2-methoxy-5-nitrobenzyl bromide (MNB), dimethyl sulfonium bromide (DMSB), dicyclohexylcarbodiimide (DCC), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), Woodward's reagent K (WK), diethylpyrocarbonate (DEPC), *N*-ethylmaleimide (NEM), dithiobisnitrobenzoate (DTNB), iodoacetate, iodoacetamide, *p*-chloromercuribenzoate (PCMB), dinitrofluorobenzene (DNFB), trinitrobenzenesulfonic acid (TNBS), phenylglyoxal, *N*-acetylimidazole (NAI), *N*-butyrylimidazole (NBI), phenylacetate, paraoxon, terbium chloride, *L*-homoarginine, and paranitrophenyl phosphate.

Table 2: Effects of Group-Selective Reagents on PON1 Activity

| reagent[a] | [reagent]/[PON1] (mol/mol) | group selectivity[b] | inactivation (%) | buffer |
|---|---|---|---|---|
| NBS | 500 | Trp | 100 | *c* |
| HNB | 5000 | Trp | 30 | *c* |
| MNB | 5000 | Trp | 30 | *c* |
| DMSB | 5000 | Trp | <20 | *c* |
| DCC | 1000 | Asp, Glu | 100 | *c* |
| EDC | 1000 | Asp, Glu | 100 | *c* |
| EEDQ | 1000 | Asp, Glu | 100 | *c* |
| WK | 10000 | Asp, Glu | 30 | *c* |
| DEPC | 5000 | His | 50 | *d* |
| NEM | 10000 | Cys | <20 | *e* |
| DTNB | 10000 | Cys | 30 | *e* |
| iodoacetate | 10000 | Cys | <20 | *e* |
| iodoacetamide | 10000 | Cys | <20 | *e* |
| PCMB | 10000 | Cys | <20 | *e* |
| DNFB | 5000 | Arg, Lys | <20 | *e* |
| TNBS | 5000 | Arg, Lys | <20 | *e* |
| phenylglyoxal | 5000 | Arg, Lys | <20 | *e* |
| NAI | 5000 | Tyr | <20 | *e* |
| NBI | 5000 | Tyr | <20 | *e* |

[a] Abbreviations used: see Materials and Methods section. [b] The most likely side chains modified under the conditions of the experiment. [c] 20 mM MES/NaOH, pH 6.0. [d] 20 mM MOPS/NaOH, pH 7.0. [e] 50 mM HEPES/NaOH, pH 8.75.

Diazoxon and chlorpyrifos-oxon were kindly provided by Novartis (Greensboro, NC) and DowElanco (Indianapolis, IN), respectively. *Organophosphate compounds (paraoxon, diazoxon, and chlorpyrifos-oxon) are nerve poisons that were handled with care. Waste was inactivated by treatment with 0.1 M NaOH solution.*

$^{45}CaCl_2$ (15 to 30 mCi/mg) was purchased from NEN Life Science Products (Boston, MA).

*Purification of Natural Human and Rabbit PON1*. Human PON1 was highly purified from frozen serum provided by the Centre de Transfusion sanguine (Lyon-Miribel, France) according to the two-step protocol of Gan et al. (*20*): affinity chromatography on Blue-agarose and anion exchange chromatography on DEAE-sepharose. The purified enzyme showed two or three bands (37−45 kDa), corresponding to glycoforms (*19*), after SDS−PAGE. It had a specific activity of about 1000 units/mg with phenylacetate as substrate (a unit of activity is micromole of substrate hydrolyzed per min at 25 °C and pH 8.0). Frozen rabbit serum was provided by Animalerie of Centre de Recherches du Service de Santé des Armées (La Tronche, France). Rabbit PON1 was purified according to the same procedure as for human PON1. It had a specific activity of 800 units/mg with phenylacetate as substrate.

*Group-Selective Labeling.* Inactivation of purified human PON1 with different group-selective labeling reagents (Table 2) was carried out according to current procedures (*24*). The pH of the reaction as well as the molar excess of reagent over the enzyme was carefully designed to optimize the group selectivity of each reagent. Stock solutions of group-selective reagents were made in methanol or water. At initial time, the incubation medium (1 mL final volume) consisted of PON1 (50 $\mu$L of a 1−5 $\mu$M stock solution in 50 mM Tris/HCl buffer, pH 8, containing 1 mM $CaCl_2$ and 0.1% Triton X-100) diluted in an appropriate buffer and an excess (10−10000-fold) of group-selective reagent, at 20 °C. Because methanol (>5%) affects the enzyme activity (*25*), no more than 5% methanol was present in the incubation

medium. Aliquots were withdrawn at specific time intervals, and the enzyme residual activity was measured with phenylacetate as substrate.

*Protection Against Chemical Inactivation.* PON1 protection against inactivation by NBS, DCC, and DEPC was studied by preincubating the enzyme with either $CaCl_2$ (5 mM final) or phenylacetate (5 mM final), 1 min before starting the chemical inactivation reaction, followed by measuring the enzyme residual activity.

*Mutagenesis of Human PON1.* In all PCR reactions described below, human PON1 cDNA cloned in pGS plasmid (Scios Nova, Mountain View, CA) or in pcDNA3 plasmid (Invitrogen, Carlsbad, CA) was used as template. The encoded amino acids in positions 54 and 191, corresponding to polymorphic sites in human PON1 (*9*), were Met and Gln, respectively.

*Incorporation of an Oligonucleotide Sequence Coding for a 6-Histidine Residue Tag at the 5′ or 3′ End of PON1 cDNA.* A PCR-based reaction was designed to create a 6-histidine residue tag at the N- or the C-terminal end of PON1.

*Site-Directed Mutagenesis.* The single replacement of amino acids was achieved by PCR-based site-directed mutagenesis of human PON1 cDNA following the protocol of Chen and Przybyla (*26*). The primers used to introduce mutations in PON1 cDNA were synthesized by the University of Nebraska Molecular Biology Core Facility. PCR products, purified using a QIAquick-spin PCR purification kit (Qiagen), were digested with *Hin*dIII and *Apa*I restriction enzymes (Promega, WI) and ligated into pGS or pcDNA3 expression plasmids. These plasmids were propagated in *Escherichia coli* HB101 cells, then purified using the Qiagen plasmid maxi kit. The complete nucleotide sequence of each cloned mutant was determined by the University of Nebraska Molecular Biology Core Facility.

*Transient Expression of Recombinant PON1 Wild-Type and Mutants.* The highly transfectable 293T/17 human embryonic kidney cell line (ATCC No CRL 11268) was used with permission of David Baltimore (*27*). Cells in Dulbecco's Minimal Essential Medium (DMEM) with 5% fetal calf serum were cotransformed, using calcium phosphate−DNA precipitates, with pGS−PON1 wild-type or mutant and the reporter vector pSEAP2 (Clontech) expressing secreted alkaline phosphatase. At least three 100 mm dishes of 293T cells were used per mutant or wild-type PON1. Four days after the cell transformation, there was enough transiently expressed PON1 (20−100 $\mu$g in 10 mL) and alkaline phosphatase reporter to determine the catalytic parameters $k_{cat}$ and $K_m$ with phenylacetate, diazoxon, chlorpyrifos-oxon, and paraoxon as substrates.

*Spectrofluorimetry.* Fluorescence spectra of PON1 were recorded on a Perkin-Elmer 650-40 fluorimeter, at 25 °C. The excitation and emission band-passes were set to 2 nm. The excitation and emission paths were 2 and 10 mm, respectively.

*Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting.* SDS−PAGE was carried out according to Laemmli (*28*). All transiently expressed mutants and wild-type PON1, in DMEM medium, were applied to 5−20% gradient gels and electroblotted onto a PVDF membrane (Immobilon-P, Millipore). The membrane was first incubated in Tris buffer saline (TBS) (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 0.2% Tween and 5% dry milk at 25 °C for 1 h, then incubated with the F41F2-K monoclonal anti-human PON1 antibody (*29*) at 25 °C for at least 2 h. The membrane was successively washed (5 min for each step) in several TBS buffers containing the following: no milk, no Tween; 5% milk, 0.2% Tween; 5% milk, 3% Tween; no milk, no Tween; no milk, 0.2% Tween; and 5% milk, no Tween. It was then incubated at 25 °C for 2 h with an anti-mouse Ig, horseradish peroxidase-linked antibody from sheep (Amersham, Airlington Heights, IL) and washed again as before. The peroxidase activity was revealed with a chemiluminescent detection kit as recommended by the supplier (LumiGLO substrate kit, Kirkegaard and Perry Laboratories, Gaithersburg, MD).

*$^{45}Ca^{2+}$ Binding to PON1.* We performed native, nondenaturating gel electrophoresis to determine $^{45}Ca$ binding to PON1. Human and rabbit PON1 samples (20 and 2 $\mu$g, respectively) containing 0.1 mM $CaCl_2$ and 20 mM MES/NaOH buffer, pH 6.8, were incubated overnight with 5 mM $^{45}Ca^{2+}$. The 200 $\mu$L samples were loaded in a 5−20% gradient polyacrylamide gel containing 0.05% Triton X-100. The electrophoresis was conducted at 25 °C, for 24 h at constant voltage (100 V). This allowed the unbound $^{45}Ca^{2+}$, in excess in the samples, to accumulate at the cathode, which resulted in a clear background. The gel was quickly washed in distilled water, then dried before being exposed for one to two hours to a Phosphor Screen that was analyzed by a Storm 860 phosphorimager (Molecular Dynamics, Sunnyvale, CA).

*Enzyme Activity Assays.* Enzyme assays were performed at 25 °C, using a Shimadzu UV-160A spectrophotometer. Units of activity are micromoles of substrate hydrolyzed per min.

*Arylesterase Activity.* The arylesterase activity of PON1 was determined with phenylacetate (0.1−5 mM) as substrate in 20 mM Tris/HCl buffer pH 8.0, containing 1 mM $CaCl_2$, as previously described (*20*).

*OP-Hydrolase Activity.* The hydrolysis of diazoxon (20−700 $\mu$M) and chlorpyrifos-oxon (20−800 $\mu$M) was measured in 100 mM Tris/HCl, pH 8.5, buffer containing 2 M NaCl and 2 mM $CaCl_2$, as previously reported (*12*, *30*).

The hydrolysis of paraoxon (0.1−2 mM) was measured in 50 mM glycine/NaOH, pH 10.5, buffer containing 1 mM $CaCl_2$ according to Gan et al. (*20*).

*Alkaline Phosphatase Activity.* Alkaline phosphatase activity was determined according to a microtiter plate assay (*31*), slightly modified to adapt it to a cuvette assay. Secreted recombinant PON1 (500 $\mu$L) in cell culture medium was heated for 5 min at 65 °C to inactivate endogenous phosphatases, then mixed with 500 $\mu$L of 2 × SEAP buffer (10.51 g of diethanolamine, 50 $\mu$L of 1 M $MgCl_2$ and 226 mg of *L*-homoarginine in 50 mL of water). The alkaline phosphatase activity was measured by following the absorbance change with time at 405 nm after adding 40 $\mu$L of substrate stock solution (120 mM paranitrophenyl phosphate in 1 × SEAP) to 400 $\mu$L of the previous mixture and 600 $\mu$L of 1 × SEAP.

*Determination of the Catalytic Constants $k_{cat}$ and $K_m$.* The catalytic constants $V_{max}$ and $K_m$ were determined with the software KaleidaGraph version 3.0 using the Lineweaver−Burk double reciprocal plot. Seven substrates' concentrations were used to build plots. Determinations were performed at least in triplicate.

Since we could not accurately determine the amount of recombinant PON1 expressed by the 293T cells, we calculated $k_{cat}$ apparent values as the ratio of $V_{max}$ of PON1 over the alkaline phosphatase activity secreted in the culture medium. The $V_{max}$ of SEAP mainly depends on (a) the efficiency of cells transformation, (b) the number of cells per dish, and (c) the rate of protein secretion. The $V_{max}$ of secreted PON1 activity depends on the same factors as for SEAP, and on two other parameters: first, the specific rate of secretion of wild-type and mutants PON1 (that we first assumed to be the same[2]), and second, the $k_{cat}$ of PON1. When calculating the ratio PON1/SEAP, we thus standardized wild-type and mutants PON1 activity for the factors affecting SEAP activity (a, b, c). Differences in PON1/SEAP ratio values for wild-type and mutants PON1 directly reflect differences in $k_{cat}$.

## RESULTS

*Screening of the Effects of Reagents with Different Group Selectivity on PON1 Activity.* Among the reagents used to chemically modify PON1, only a few reduced the enzyme activity by more than 20% after a 30 min reaction (Table 2). PON1 was not significantly inactivated by Tyr-, Arg-, and Lys-selective reagents, and was partially inactivated by Cys- and His-selective reagents. By contrast, PON1 was totally inactivated by the Trp- and Asp/Glu-selective reagents NBS and DCC, EDC, and EEDQ, respectively.

Interestingly, PON1 activity was affected differently by reagents of the same group selectivity. For instance, the hydrophobic carboxylate-selective reagents, DCC, EDC, and EEDQ, more effectively inactivated PON1 than the hydrophilic reagent WK. This suggests that the chemical inactivation of PON1, and very likely the active site accessibility of this enzyme, are dependent on both hydropathy and structural characteristics of the reagents. Given these results, we chose to further study PON1 chemical inactivation caused by Trp-, His-, and carboxylate group-selective reagents.

*PON1 Inactivation by NBS.* NBS stock solutions (20 mM) were prepared in water. The effect of NBS on PON1 was examined under mild acidic conditions, pH 6.0−6.5, because more acidic conditions (pH < 4.0) generally recommended for NBS modification resulted in PON1 inactivation. We found that PON1 was significantly inactivated at pH < 6.0, probably as a result of a competition between calcium and protons for the calcium-binding sites.

*Kinetics of Inactivation by NBS.* As shown in Figure 1A, PON1 inactivation by NBS is a time-dependent process. At a given enzyme concentration, the rate of inactivation was dependent on the NBS concentration: at pH 6.5, a 250-fold molar excess of NBS over the enzyme concentration was enough to allow 50% inactivation of PON1 after about 15 min of reaction. Semilog plots of PON1 residual activity against time (Figure 1B) show that PON1 inactivation by NBS is a biphasic process.

*Selectivity of the Chemical Modification.* Although NBS is considered to be selective for Trp residues, other functional groups, such as histidine, cysteine, and tyrosine (*24*), may react with NBS, especially at pH values close to neutrality. If these reactions have occurred for PON1, then lowering the pH should slow the first-order rate constant for the inactivation process. With a 500-fold molar excess of NBS over PON1, the first-order inactivation constant at pH 6 was found to be 10-fold higher than at pH 6.5, indicating that PON1 inactivation by NBS is directly related to the chemical modification of Trp residue(s).

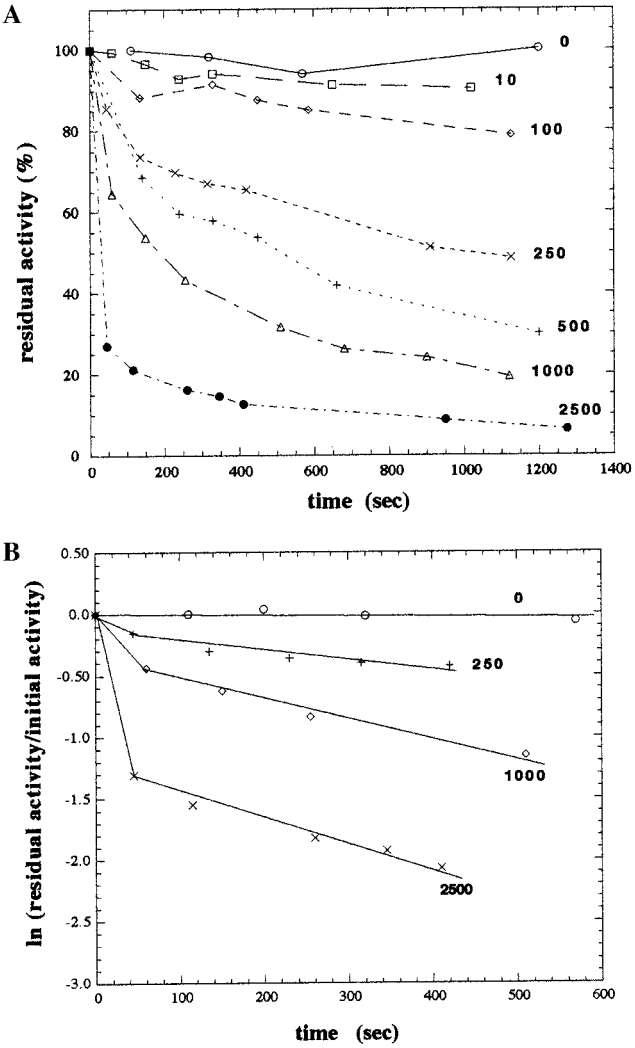


FIGURE 1: Kinetics of inactivation of human PON1 by NBS. (A) Purified human PON1 (50 $\mu$L of 1 $\mu$M solution) was incubated with NBS (0−2500-fold molar excess over the enzyme, as indicated on the curves) in 20 mM MES/NaOH buffer, pH 6.5, in 1 mL final volume. Aliquots were removed as a function of time, and the residual activity was determined with phenylacetate. (B) Semilog plots of data corresponding to Figure 1A.

*Protection of PON1 against Chemical Inactivation by NBS.* As indicated in Table 3, PON1 was partially protected against NBS inactivation by calcium ions, and even better protected by phenylacetate. This suggests that the modified Trp residue(s) is (are) located in the PON1 active center and calcium-binding sites.

*Conformational Effects of the Chemical Modification.* The observed loss of PON1 activity due to Trp oxidation by NBS may result from either a selective modification of active site residues or a conformational change owing to the chemical modification of residue(s) remote from the active site. A shift

[2] This hypothesis was qualitatively checked by western blot analysis: when standardized for SEAP activity, the intensity of the bands corresponding to the wild-type and most of the mutant PON1 was similar. Obviously, this was not the case for the C41A, C352A, C41A−C352A, and H154N mutants (see Results).

Table 3: Protection of PON1 against Chemical Modification[a]

| reagent | group selectivity | residual activity (%) after 30 min reaction | | |
|---|---|---|---|---|
| | | no protection[b] | +Calcium[c] | +phenylacetate[d] |
| NBS[e] | Trp | 20 | 55 | 85 |
| DCC[f] | Asp, Glu | 0 | >90 | 0 |
| DEPC[g] | His | 50 | 50 | 50 |

[a] The chemical inactivation of PON1 was started after a 1 min preincubation of the enzyme with either 0.05 mM $CaCl_2$, 5 mM $CaCl_2$, or 5 mM phenylacetate. [b] $CaCl_2$ (0.05 mM). [c] $CaCl_2$ (5 mM). [d] Phenylacetate (5 mM). [e] Molar excess of reagent over the enzyme was 500−fold, in 20 mM MES/NaOH, pH 6.5. [f] Molar excess of reagent over the enzyme was 500−fold, in 20 mM MES/NaOH, pH 6.0. [g] Molar excess of reagent over the enzyme was 5000−fold, in 20 mM HEPES/NaOH, pH 7.0.

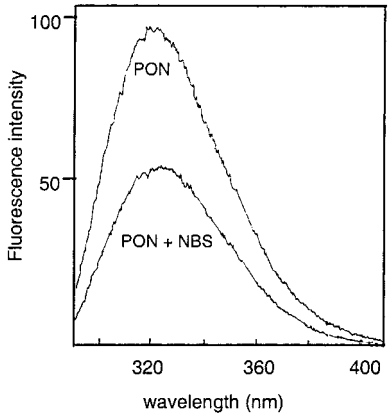


FIGURE 2: Fluorescence emission spectra of human PON1. The fluorescence emission spectra of human PON1 (2.5 $\mu$M, in 20 mM MES/NaOH buffer, pH 6.5), in the native state and in the NBS-inactivated state, are shown after excitation at 280 nm.

in the PON1 intrinsic maximum fluorescence emission was expected if extensive conformational change had occurred. As seen in Figure 2, PON1 was found to lose Trp fluorescence upon NBS treatment with no shift in the maximum fluorescence emission compared to that of the native enzyme. This result clearly indicates that one or more Trp were oxidized by NBS and that no extensive conformational change of the enzyme occurred. Modification by NBS does not introduce a bulky group into the enzyme structure that might have caused inactivation by steric hindrance in the 3D structure, since the only change is the addition of an oxygen to the indole ring (*24*). Inactivation of PON1 by NBS oxidation of reactive Trp residues may arise from changes in the hydrophobicity or $\pi$-interaction ability of either the catalytic or the substrate-binding site as has already been shown for other enzymes such as lysozyme (*32*) and transcarboxylase (*33*).

*PON1 Inactivation by DCC.* DCC stock solutions (100 mM) were prepared in methanol. PON1 inactivation by DCC is a time-dependent monophasic process. Figure 3 shows representative semilog plots of PON1 % residual activity against time, with various DCC over PON1 molar ratio. The second-order rate constant for inactivation was calculated from a plot of the slopes in Figure 3 versus concentration of DCC. PON1 inactivation by DCC is highly significant as reflected by the second-order rate constant value of 27 $M^{-1}$ $s^{-1}$. The reaction order for inactivation with respect to DCC concentration was calculated from the slope of the double logarithmic plot of the apparent first-order rate constant

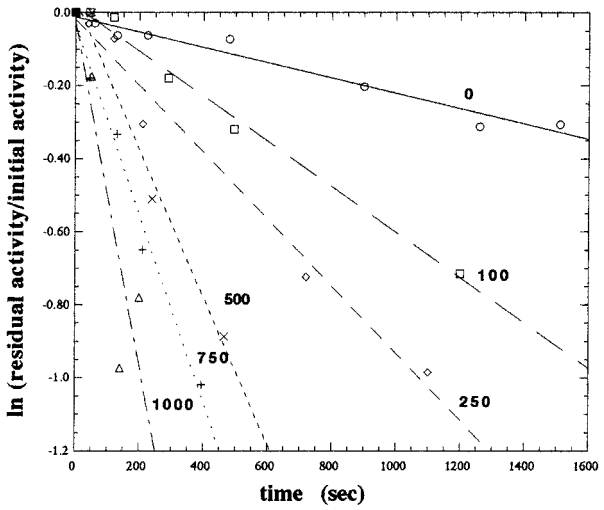


FIGURE 3: Kinetics of inactivation of human PON1 by DCC. Purified human PON1 (50 $\mu$L of 1 $\mu$M solution) was incubated with DCC (0−1000-fold molar excess over the enzyme, as indicated on the curves) in 20 mM MES/NaOH buffer, pH 6.0, in 1 mL final volume. Aliquots were removed as a function of time, and the residual activity was measured immediately.

against the reagent concentration. It yielded a value of about 1, indicating that only one reactive essential residue was modified by DCC per mole of enzyme active sites. Furthermore, inactivation by DCC was pH-dependent with a maximal effect at pH 6.0. For example, the first-order rate constant for the inactivation was 20-fold higher at pH 6.0 than at pH 7.4. This result strongly supports the conclusion that DCC selectively modifies a carboxylate amino acid residue in PON1.

As indicated in Table 3, phenylacetate did not protect PON1 from chemical inactivation by DCC. By contrast, 5 mM calcium chloride achieved greater than 90% protection. This suggests that the essential carboxylic amino acid residue modified by DCC does not interact with the substrate but is part of the PON1 calcium-binding site.

PON1 inactivated by DCC did not have an altered enzyme fluorescence spectrum, indicating that the Trp environment and the overall protein conformation were not significantly altered by the chemical modification.

*PON1 Inactivation by DEPC.* Incubation of PON1 with various concentrations of DEPC at pH 7.0 resulted in a time-dependent loss of enzymatic activity. Although the spontaneous DEPC degradation (*34*) was taken into account, semilog plots of PON1 % residual activity against time (Figure 4) were nonlinear, suggesting that at least two amino acid residues were modified by DEPC. The difference spectroscopy spectrum of DEPC-modified and native PON1 showed an absorbance maximum at 242 nm, characteristic of *N*-carbethoxyhistidine (*34*, *35*) (result not shown). The change in absorbance at 242 nm was directly correlated with the progressive loss of enzyme activity. This result indicates that PON1 inactivation by DEPC is at least partially associated with the chemical modification of one or more His residues. Incubation of the DEPC-modified enzyme with hydroxylamine (0.5 M for 30 min), a reagent known to release *N*-carbethoxyl from His residues, did not result in a significant reactivation of enzyme activity. However, compared to untreated PON1, the protein fluorescence emission spectrum of the DEPC-modified PON1 was not affected,

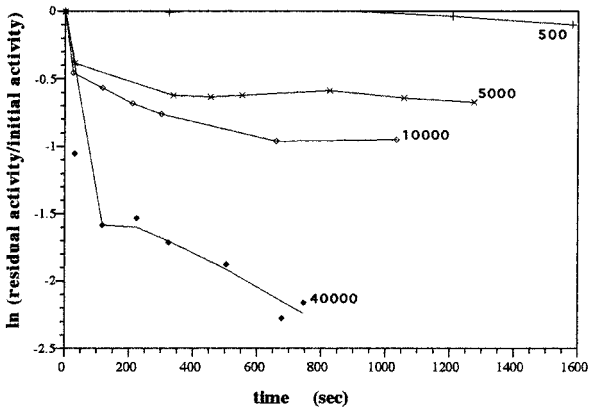


FIGURE 4: Kinetics of inactivation of human PON1 by DEPC. Purified human PON1 (50 $\mu$L of 1 $\mu$M solution) was incubated with DEPC (500−40000-fold molar excess over the enzyme, as indicated on the curves) in 20 mM MOPS/NaOH buffer, pH 7.0, in 1 mL final volume. Aliquots were removed as a function of time, and the residual activity was determined. The *x*-axis has been corrected for decomposition of DEPC (*35*).

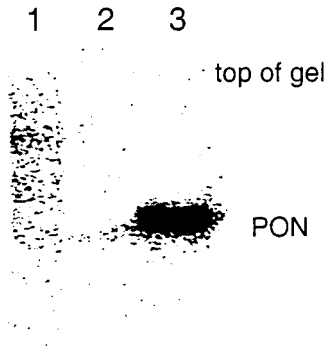


FIGURE 5: $^{45}Ca^{2+}$-binding assay. Purified human acetylcholinesterase (70 $\mu$g, lane 1), human PON1 (20 $\mu$g, lane 2), and rabbit PON1 (2 $\mu$g, lane 3) were incubated overnight with 5 mM $^{45}Ca^{2+}$, then loaded on a 5−20% gradient polyacrylamide gel. The electrophoresis was conducted for 24 h at constant voltage (100 V).

indicating that Trp residues were not covalently modified by DEPC. Finally, neither phenylacetate nor calcium ions protected PON1 from inactivation by DEPC (Table 3), suggesting that the modified amino acids were not part of the PON1 active center and catalytic calcium-binding site.

*$^{45}$Calcium-Binding to Native PON1.* Different electrophoretic methods have been developed to detect proteins that bind $Ca^{2+}$ (*36*−*38*). However, when applied to PON1, these methods were either found too drastic for PON1 stability or had several drawbacks regarding sensitivity as well as cost and time consumption. For instance, as opposed to EF-hand calcium-binding proteins (*36*), PON1 did not bind $^{45}Ca^{2+}$ after an SDS−PAGE and transfer to a PVDF membrane. Instead, we developed a simple semiquantitative and sensitive calcium-binding assay based on: (a) exchange of the "cold" calcium ions bound to PON1 by an excess of $^{45}Ca^{2+}$, and (b) elimination of the nonspecifically bound positively charged $^{45}Ca^{2+}$ from the negatively charged PON1 by nondenaturating gel electrophoresis. As seen in Figure 5, the assay for $^{45}Ca^{2+}$ binding to PON1 was very sensitive for rabbit PON1 but relatively insensitive for human PON1. As much as 20 $\mu$g of human PON1 was barely detected (lane 2, Figure 5), although under the same conditions, this assay allowed the detection of 2 $\mu$g of rabbit PON1 (lane 3), 0.5

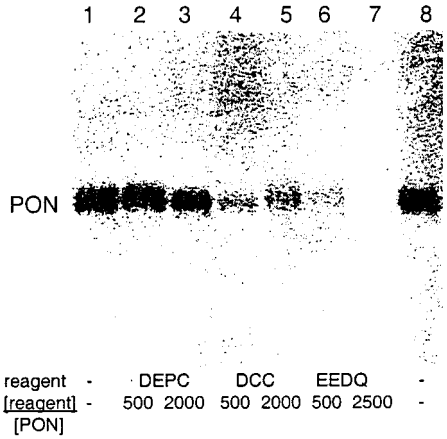


FIGURE 6: $^{45}Ca^{2+}$ binding to chemically modified PON1. Purified rabbit PON1 (4 $\mu$g) was reacted for 1 h with the group-selective reagents DEPC (in 20 mM MOPS/NaOH buffer, pH 7.0), DCC (in 20 mM MES/NaOH buffer, pH 6.0), and EEDQ (in 20 mM MES/NaOH buffer, pH 6.0), and then incubated overnight with $^{45}Ca^{2+}$ (5 mM, 30 mCi/mg). The samples were loaded on a 5−20% gradient polyacrylamide gel. The electrophoresis was conducted for 24 h at constant voltage (100V). Lanes 1 and 8 contain unmodified native enzyme; lanes 2 and 3, DEPC-treated PON1; lanes 4 and 5, DCC-treated PON1; and lanes 6 and 7, EEDQ-treated PON1.

$\mu$g of calmodulin (not shown), and 70 $\mu$g of human acetylcholinesterase (lane 1). The result that purified rabbit PON1 was detected with good sensitivity confirms previous data that indicated a higher affinity of rabbit PON1 for $Ca^{2+}$ as compared to human PON1 (*22*). Considering the better calcium-binding affinity of rabbit PON1, we decided to use the rabbit enzyme to study the effects of the chemical modifications on $^{45}Ca^{2+}$ binding to PON1. We intend to use the rabbit results as an indication of the structure of the human PON1. This is justified given that human and rabbit PON1 show 85% identity in amino acid sequence (*19*), hydrolyze the same substrates, and are similarly inactivated by DCC and NBS.

*Effect of Chemical Modifications on $^{45}$Calcium Binding to PON1.* As shown in Figure 6, treatment of rabbit PON1 with DEPC did not affect the binding of $^{45}Ca^{2+}$ ions. By contrast, NBS (not shown), DCC, and EEDQ strongly reduced or even prevented PON1 from binding $^{45}Ca^{2+}$ ions, suggesting that the Trp, Asp, or Glu amino acids covalently modified are located in the calcium-binding site(s).

*Binding of $Tb^{3+}$ Ions to PON1.* When 0.1 mM $TbCl_3$ was added to PON1 in the presence of 1 mM $CaCl_2$, PON1 activity fell to 0. This suggests that $Tb^{3+}$ binds in the calcium-binding site(s) of PON1. The kinetic data indicated that the apparent affinity of PON1 for terbium is at least 100 times higher than for calcium. As seen in Figure 7, $Tb^{3+}$ ions competitively displaced $^{45}Ca^{2+}$ ions from the same metal-binding sites on PON1. Upon substitution of $Ca^{2+}$ with $Tb^{3+}$ and excitation at 282 nm, the PON1−$Tb^{3+}$ complex exhibited a characteristic fluorescence emission maximum at 545 nm (Figure 8) indicative of the presence of a Trp and/or a Tyr residue close to the terbium-binding site(s) (*39*−*42*). The PON1−$Tb^{3+}$ emission was reversed by the addition of the terbium-chelating agent EDTA, but not by the addition of 100-fold molar excess $Ca^{2+}$ ions.

*Effect of the Chemical Modifications on Terbium−PON1 Fluorescence.* PON1 covalent modification in 20 mM MES/

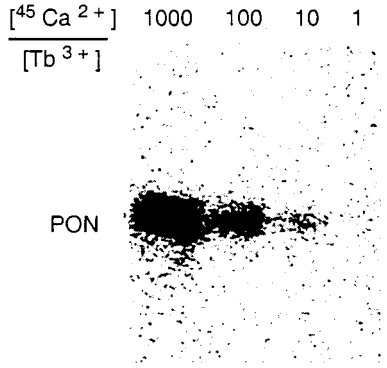


FIGURE 7: Competitive displacement of $^{45}Ca^{2+}$ by $Tb^{3+}$ ions on rabbit PON1. Purified rabbit PON1 (4 $\mu$g) was incubated overnight in 20 mM MES/NaOH buffer, pH 6.0, containing $^{45}Ca$ (5 mM). Increasing amounts of $Tb^{3+}$ ions were added to the incubation medium to give final concentrations of 5 $\mu$M, 50 $\mu$M, 500 $\mu$M, and 5 mM. The ratios of $^{45}Ca$ ion concentration to Tb ion concentration were thus 1000, 100, 10, and 1. The samples were loaded on a 5−20% gradient polyacrylamide gel, and the electrophoresis was conducted for 24 h at constant voltage (100V).

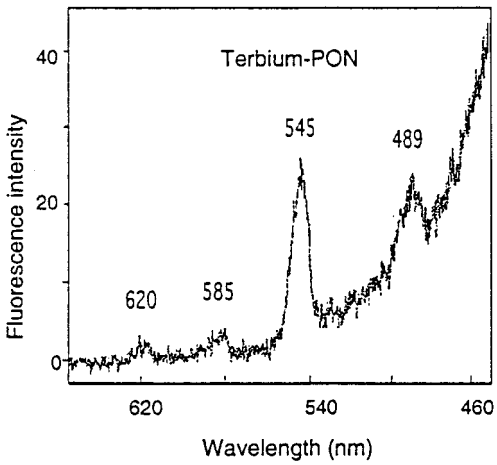


FIGURE 8: Terbium-PON1 fluorescence emission spectrum. Purified human PON1 (5 $\mu$M) in 20 mM MES/NaOH buffer, pH 6.5, containing 0.1 mM $CaCl_2$, 0.1 mM $TbCl_3$, and 20% glycerol was excited at 282 nm. A 355 nm short wavelength cutoff filter was used in the emission path to reduce the intense protein fluorescence emission as a consequence of the 282 nm excitation. The four maxima at 489, 545, 585, and 620 nm are characteristic of $Tb^{3+}$ emission, sensitized after an energy transfer from a nearby aromatic amino acid.

NaOH buffer, pH 6, by the Asp/Glu- and His-selective reagents, DCC (1000-fold molar excess) and DEPC (10000-fold molar excess), did not quench the PON1−$Tb^{3+}$ fluorescence. In contrast, the Trp-selective reagent NBS (500-fold molar excess) quenched fluorescence. This suggested that a Trp−$Tb^{3+}$ energy relay system is involved in the latter emission, and that Trp is near the metal-binding site.

*Effect of Adding a 6-Histidine Residue Tag on Human PON1 Activity.* The incorporation of a 6-histidine residue tag either on the C-terminal (6HCPON) or on the N-terminal end of wild-type PON1 lowered $k_{cat}$ by 30% and 70%, respectively, while the $K_m$ of the 2 His-tag recombinant enzymes was not significantly different from that of recombinant wild-type PON1. Our future aim is to purify wild-type and mutant PON1 on a 6-histidine residue tag affinity column. Given these results, we chose to use the 6HCPON

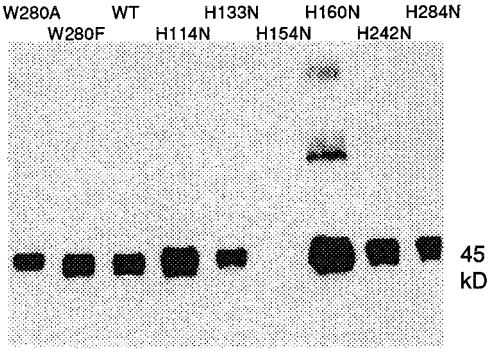


FIGURE 9: Western blot. A 5−30% gradient SDS−PAGE, of wild-type (WT) and mutants of human PON1, W280A, W280F, H114N, H133N, H154N, H160N, H242N, and H284N, was transferred to PVDF membrane and hybridized with a monoclonal anti-human PON1 antibody. Each lane of the gel was loaded with 50 $\mu$L of recombinant PON1 in culture medium, plus 10 $\mu$L of SDS sample buffer (SDS 20%/$\beta$-mercaptoethanol/glycerol, 5:2:3).

cDNA as a template in the PCR reaction to construct the mutated cDNA.

*Importance of PON1 Disulfide Bond and Glycosylation Sites.* Our results (see Table 4) confirmed that replacement of the free cysteine residue (C283) with alanine did not markedly affect PON1 arylesterase activity as already shown by Sorenson et al. (*18*). On the other hand, substitution of the disulfide-linked C41 and/or C352 with Ala led to PON1 inactivation and significantly reduced PON1 secretion by 293T cells as evidenced by western blot analysis (not shown). Interestingly, mono *N*-deglycosylated PON1, N252Q, or N323Q mutant, as well as the totally *N*-deglycosylated enzyme, N252Q/N323Q double mutant, was secreted by the 293T cells; additionally, their activity was reduced less than 50% compared to the wild-type PON1.

*Design of Other Mutations.* On the basis of the group-selective labeling results, we chose to systematically alter by site-directed mutagenesis the Trp, His, and Asp/Glu residues conserved among the mammalian PON1 (*17*) and to study the effects of these single substitutions on human PON1 activity. As a first approach, 4 Trp residues (out of 4 conserved) and 18 acidic amino acids (out of 35 conserved) were changed to Ala, whereas 10 out of 12 conserved His residues were substituted with Asn to minimize structural alterations in the enzyme conformation. Moreover, we investigated the effects on PON1 activity of 3 point mutations (W280L, H154Y, and H242K) corresponding to natural mutations found in PON2 or PON3 (*17*).

*Expression of Wild-Type PON1 and Mutants by 293T Cells.* Expression of wild-type and mutant PON1 was checked by activity assays (next section) and western blot analysis. As shown in Figure 9, H154N PON1 was not detected by SDS−PAGE immunoblot analysis of the cell culture medium. All other inactive mutants were detected on a Western blot. However, the H154N mutant was detected by western blot of cell lysis medium. The H154N mutation may have affected the folding process of the protein, and consequently its secretion. It should be noted that the monoclonal anti-human PON1 antibody used in this study did not react with the purified rabbit PON1.

*Kinetic Analysis of Wild-Type and Mutant PON1 (Table 4).* The catalytic activity of wild-type PON1 and mutants

Table 4: Catalytic Parameters of the PON1 Mutants Expressed as Percent of Wild-Type PON1 for the Hydrolysis of Phenylacetate, Diazoxon, Chlorpyrifos-oxon, and Paraoxon[a]

| | phenylacetate | | diazoxon | | chlorpyrifos-oxon | | paraoxon | |
|---|---|---|---|---|---|---|---|---|
| PON1 | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}$ |
| W193A | 113 | 37 | 138 | 39 | 151 | 47 | nd | nd |
| W193F | 139 | 112 | 110 | 88 | 155 | 137 | nd | nd |
| W201A | 110 | 34 | 136 | 34 | 108 | 34 | nd | nd |
| W201F | 129 | 181 | nd | nd | 118 | 211 | nd | nd |
| W253A | 113 | 214 | 93 | 199 | 121 | 249 | nd | nd |
| W253F | 132 | 261 | 164 | 360 | 163 | 290 | nd | nd |
| W280A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| W280L | 64 | 23 | nd | nd | 156 | 24 | nd | nd |
| W280F | 148 | 94 | 81 | 56 | 166 | 94 | nd | nd |
| H114N | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| H133N | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| H154N | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| H154Y | 100 | 74 | 94 | 67 | 91 | 69 | nd | nd |
| H160N | 122 | 51 | 137 | 44 | 116 | 43 | 98 | 72 |
| H183N | 154 | 51 | nd | nd | nd | nd | nd | nd |
| H242N | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| H242K | 106 | 96 | 85 | 81 | 171 | 142 | nd | nd |
| H245N | 100 | 54 | 116 | 32 | 102 | 28 | 132 | 73 |
| H250N | 104 | 56 | 71 | 58 | 75 | 61 | 111 | 82 |
| H284N | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| H347N | 228 | 57 | 134 | 12 | 229 | 15 | nd | nd |
| E32A | 108 | 19 | 108 | 19 | 108 | 20 | nd | nd |
| E48A | 126 | 55 | 107 | 51 | 87 | 41 | nd | nd |
| E52A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| D53A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| E55A | 107 | 85 | 168 | 77 | 106 | 114 | nd | nd |
| D88A | 108 | 23 | 101 | 21 | 141 | 26 | nd | nd |
| D107A | 72 | 47 | nd | nd | 68 | 39 | nd | nd |
| D121A | 94 | 78 | nd | nd | 81 | 85 | nd | nd |
| D123A | 90 | 24 | 143 | 33 | 60 | 17 | nd | nd |
| E147A | 78 | 73 | nd | nd | 62 | 72 | nd | nd |
| D168A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| D182A | nd | 5 | nd | <5 | nd | nd | nd | nd |
| E194A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| D230A | 71 | 20 | 142 | 33 | nd | nd | nd | nd |
| E238A | 181 | 52 | nd | nd | nd | nd | nd | nd |
| D268A | nd | <1 | nd | <5 | nd | <5 | nd | <5 |
| D273A | 100 | 78 | 126 | 73 | 108 | 72 | nd | nd |
| D278A | 123 | 12 | nd | nd | 183 | 5 | nd | nd |
| C283A | 82 | 105 | nd | nd | nd | nd | nd | nd |
| C41A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| C352A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| C41A/C352A | nd | <5 | nd | <5 | nd | <5 | nd | <5 |
| N252Q | 77 | 99 | 128 | 130 | 145 | 148 | nd | nd |
| N323Q | 54 | 77 | 121 | 104 | 67 | 71 | nd | nd |
| N252Q/N323Q | 103 | 61 | 105 | 51 | 106 | 56 | nd | nd |

[a] Each indicated value corresponds to the average of at least 3 different determinations, with a coefficient of variation less than 20%. nd, not determined. The $K_m$ values for wild−type human PON1 ($n = 6$) are 0.42 ± 0.07 mM (phenylacetate), 0.30 ± 0.04 mM (diazoxon), 0.36 ± 0.04 mM (chlorpyrifos−oxon), and 0.54 ± 0.04 mM (paraoxon).The $k_{cat}$ values for native human PON1, calculated from Davies et al. (*12*), are 1236 $s^{-1}$ (phenylacetate), 110 $s^{-1}$ (diazoxon), 67 $s^{-1}$ (chlorpyrifos-oxon), and 3 $s^{-1}$ (paraoxon).

was assayed using phenylacetate, diazoxon, chlorpyrifos-oxon, and paraoxon as substrates. With all substrates, kinetic parameters of wild-type and, when measurable, mutant PON1 fit the Michaelis−Menten kinetics model. Values of $k_{cat}$ and $K_m$ for each mutant were compared to $k_{cat}$ and $K_m$ of the wild-type enzyme.

*Trp Mutants.* The W193A and W201A mutants exhibited $k_{cat}$ values ranging from 30% to 50% of wild-type PON1 activity depending on the substrate. The W253A mutant was 2-fold more active than wild-type. The $K_m$ values of these mutants remained relatively unaltered whatever the substrate. When the same residues were conservatively substituted with Phe, the $k_{cat}$ values either were not significantly changed, for the W193F mutant, or were 2−4 times higher, for the W201F and W253F mutants, compared to the wild-type. By contrast, the W280L and W280A mutations decreased PON1 activity about 5-fold and more than 20-fold, respectively, while the W280F mutant exhibited wild-type activity.

*His Mutants.* The conservative replacement of each of the 10 conserved His residues with Asn led to considerable changes in the enzyme activity. With respect to the wild-type enzyme, the H160N, H183N, H245N, H250N, and H347N mutants displayed reduced activity (about 10−60%), particularly with OPs as substrates. More interestingly, compared to the wild-type enzyme, activity of the H114N, H133N, H154N, H242N, and H284N mutants toward the four substrates was less than 5%. Finally, the substitutions of His in positions 154 and 242 with Tyr and Lys, residues that naturally occur in human PON3, gave nearly wild-type activity.

*Asp/Glu Mutants.* The single substitutions of Asp/Glu with Ala at positions 32, 48, 55, 88, 107, 121, 123, 147, 230, 238, and 273 did not reduce the $k_{cat}$ values of wild-type PON1 more than 5-fold and did not highly affect its affinity for phenylacetate and OPs. By contrast, the E52A, D53A, D168A, D182A, E194A, D268A, and D278A mutants exhibited $k_{cat}$ values of less than 5% of the wild-type enzyme $k_{cat}$. The activities were too low to determine $K_m$ values.

## DISCUSSION

When we started this work the only established result about the PON1 catalytic center was that the free cysteine residue in position 283 was not the active site nucleophile (*18*). Identification of the PON1 active site residues has not yet been possible for at least three reasons: (a) the 3D structure of PON1 has not been determined; (b) PON1 does not belong to a well-characterized family of proteins that could have been used as a model; (c) no active site affinity labels and mechanism-based inhibitors have been described for this enzyme.

In the present work, we identified essential amino acid residues of PON1 by adopting a double approach based on chemical modification and site-directed mutagenesis. Since calcium ions both participate in PON1 catalytic mechanism and contribute to the structural stability of the enzyme (*22*), perturbation of calcium binding may directly affect enzyme activity. Consequently, we determined whether group-selective reagents that labeled essential residues also targeted potential calcium-binding site components of PON1.

*Identification of PON1 Essential Residues.* In this study, evidence for the presence of Trp, Asp/Glu, and His residues in the PON1 active center and/or calcium-binding sites has been accumulated.

*Trp Residue(s) in the PON1 Active Center and Calcium-Binding Sites.* Selective oxidation of Trp with NBS dramatically affected PON1 activity (Table 2) and was prevented by both the substrate phenylacetate and calcium ions (Table 3), strongly suggesting that Trp residue(s) is (are) present in the PON1 active site. Moreover, upon treatment of the enzyme with NBS, $^{45}Ca$ binding to PON1 was markedly reduced, indicating that essential Trp residues are in or close to Ca-binding sites. This conclusion is supported by the observation that displacement of $Ca^{2+}$ by $Tb^{3+}$ resulted in a PON1−$Tb^{3+}$ complex which showed a fluorescence emission profile consistent with the presence of a Trp in the vicinity of the $Tb^{3+}$ (Figure 8). Further support comes from the fact that treatment with the tryptophan-selective reagent NBS caused attenuation of this Tb fluorescence. Site-directed mutagenesis suggests that Trp 280 is responsible for these observations (Table 4). The aromatic/hydrophobic character of this residue was demonstrated to be very important for the enzyme activity since the W280F mutant was fully active but the W280A and W280L mutants had their activity markedly reduced. Oxidation of Trp 280 by NBS would result in the loss of its hydrophobic/aromatic character and consequently enzyme inactivation. Similarly to Trp 131 in the bacterial PTE (*11*), Trp 280 could be involved in substrate or leaving group interaction in the PON1 active center. In this respect, it is interesting to note that, in the homologous dog PON2 as well as in human and mouse PON3, whose function and potential activity are still unknown, a Leu residue is present in position 280 (*17*). Although not tested, this could mean that the ability of PON2 and PON3 to hydrolyze OPs and arylesters is very low.

*Asp/Glu Residue(s) in the PON1 Active Center and Calcium-Binding Sites.* PON1 chemical inactivation by the Asp/Glu-selective reagent, DCC, strongly suggested that an essential Asp/Glu amino acid is present in the PON1 active site (Table 2). Since calcium ions protected PON1 from chemical inactivation by DCC (Table 3), and since $^{45}Ca^{2+}$ binding was strongly reduced upon chemical modification by DCC (Figure 6), we conclude that Asp/Glu residue(s) is (are) present in PON1 calcium-binding sites. Unlike its effect on $^{45}Ca^{2+}$ binding, chemical modification by DCC did not prevent $Tb^{3+}$ binding to PON1. This result suggests that, compared to $Ca^{2+}$ ions, Tb ions are bound by additional water molecules and amino acid side chains that have not been modified by DCC. Since carboxylic amino acids are the predominant residues in the calcium coordination spheres of proteins (*43*), several among the seven essential aspartate and glutamate residues identified by site-directed mutagenesis (E52, D53, D168, D182, E194, D268, and D278) could be ideally positioned for such a role (Table 4). Although not demonstrated, it is expected that at least some of these amino acids were modified by the Asp/Glu-selective reagents DCC, EDC, and EEDQ.

In the present work, 5 Asp residues (D88, D121, D123, D268, and D273), in putative calcium-binding loops (N77−D88, D121−N132, and D262−D273) (*18*), were individually mutated to Ala. While the D268A mutation dramatically affected PON1 activity, the 4 other mutations did not lower the wild-type activity more than 5-fold. Moreover, 6 of the 7 essential carboxylic amino acids identified in this work do not belong to the potential calcium-binding loops. This suggests that the calcium-binding residues in PON1 are dispersed in the primary structure. Besides, as demonstrated for many enzymes, certain essential carboxylic amino acids could participate in the catalytic mechanism through ionic or hydrogen-bond interactions either directly with substrate or indirectly with other residues.

Finally, much lower effects on PON1 activity were observed with the hydrophilic reagent WK compared to the hydrophobic ones, EEDQ, EDC, and DCC. This indicates that accessibility to the active site and calcium-binding sites is restricted to hydrophobic compounds as already shown for the calcium-dependent phospholipase A2 (*44*).

*His Residue(s) in the PON1 Active Center and Calcium-Binding Sites.* Site-directed mutagenesis led to the identification of 5 His residues (H114, H133, H154, H242, and H284) essential for PON1 activity (Table 4). Interestingly, conservative substitutions of His 154 and His 242 with Asn reduced wild-type activity more than 20-fold and the H154N mutation even abolished secretion by 293T cells. By contrast, the mutations H154Y and H242K, that naturally occur in PON2 and PON3 (*17*), did not significantly reduce activity and secretion by 293T cells when compared to the wild-type enzyme. This suggests that the residue in position 154 has an essential role in PON1 folding. DEPC-modified residues, among them His, are not essential for enzyme activity since we could not totally inactivate the enzyme with a reasonable excess of the reagent (Figure 4). Given that calcium ions and the substrate phenylacetate did not protect PON1 from partial inactivation by DEPC (Table 3), and that $^{45}Ca$ binding

was not reduced upon chemical modification by DEPC (Figure 6), we conclude that the DEPC-modified residues are neither in the active center nor in the Ca-binding sites. These results also suggest that DEPC has no accessibility to essential His residues identified by site-directed mutagenesis. By considering other calcium-binding proteins as models (*45*), it is tempting to attribute some putative roles to His residues. Hence, they could have an acid−base catalytic function, be involved in substrate recognition, or participate in a network of electrostatic and hydrogen-bond interactions. Such a network is known to stabilize most calcium-binding sites and maintain the ionization state of the catalytic residues (*45*−*47*).

*Importance of Glycosylation Sites and Disulfide Bond for PON1 Catalytic Activity.* Kinetic results (Table 4) and western blot analysis of the transformed 293T cells culture medium showed that glycosylation is not important for secretion and activity of recombinant PON1. By contrast, the disulfide bond was found to be very important for recombinant PON1 secretion and catalytic activity. Both results are interesting to consider in view of functional expression of PON1 in a bacterial host where glycosylation and disulfide bond formation normally found in mammalian proteins are compromised.

In conclusion, we have determined that essential Trp and Asp/Glu residues are in the calcium-binding sites and active center of PON1. We identified Trp 280, His 114, 133, 154, 242, 284, Asp 53, 168, 182, 268, 278, Glu 52,194 and Cys 41, 352 as essential residues for PON1 arylesterase and organophosphatase activity.

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