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Genetics

Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase

(organophosphatase/A-esterase/site-directed mutagenesis/high density lipoprotein/phosphotriesterase)

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ABSTRACT **For three decades, mammalian paraoxonase (A-esterase, aromatic esterase, arylesterase; PON, EC 3.1.8.1) has been thought to be a cysteine esterase demonstrating structural and mechanistic homologies with the serine esterases (cholinesterases and carboxyesterases). Human, mouse, and rabbit PONs each contain only three cysteine residues, and their positions within PON have been conserved. In purified human PON, residues Cys-41 and Cys-352 form an intramolecular disulfide bond and neither could function as an active-center cysteine. Highly purified, enzymatically active PON contains a single titratable sulfhydryl group. Thus, Cys-283 is the only probable candidate for an active-center cysteine. Through site-directed mutagenesis of the human cDNA, Cys-283 was replaced with either serine (C283S) or alanine (C283A). The expressed C283 (wild-type) enzyme was inactivated by *para*-hydroxymercuribenzoate, but the C283S and C283A mutant enzymes were not inactivated. C283A and C283S mutant enzymes retained both paraoxonase and arylesterase activities, and the K_m values for paraoxon and phenyl acetate were similar to those of the wild type. Clearly, residue Cys-283 is free in active PON, but a free sulfhydryl group is not required for either paraoxonase or arylesterase activities. Consequently, it is necessary to examine other models for the active-site structure and catalytic mechanism of PON.**

The existence of an organophosphatase in mammalian plasma was first reported nearly 50 years ago (1). Subsequent studies have shown this enzyme to be paraoxonase/arylesterase (PON; aryldialkylphosphatase, EC 3.1.8.1), an organophosphatase with broad substrate specificity, including aromatic carboxylic acid esters such as phenyl acetate [see La Du (2) for a review]. PON may have a prominent role in the metabolism of several toxic, synthetic compounds, but its physiological role and endogenous substrates remain unknown. In humans, virtually all serum PON is associated with the high density lipoprotein complex (3), and evidence is accumulating for an enzymatic, PON-mediated protection of low density lipoproteins against oxidative modification and the consequent series of events leading to atheroma formation (4–7). Because there are two common allozymic forms of PON, the intriguing possibility exists that differences in individual susceptibility to atherogenesis may be partly due to the genetic heterogeneity of this high density lipoprotein constituent. Both PON allozymes have been highly purified from serum and have distinct kinetic properties and substrate specificities (8–10). Two laboratories have independently cloned the liver PON cDNAs from human and rabbit and demonstrated that the structural basis for the human polymorphism was the presence of arginine (high PON activity, B-type allozyme) or glutamine (low PON activity, A-type allozyme) at position 191 (11–13).

Augustinsson (14, 15) suggested that PON, the carboxyesterases, and the cholinesterases were products of divergent evolution from an ancestral arylesterase but that PON utilized a cysteine residue rather than a serine residue for the nucleophilic component of its catalytic center. Cysteine was believed to be the active-center nucleophile because PON was inactivated by organic mercurial compounds that react with free sulfhydryl groups (16, 17). Augustinsson further suggested that PON's hydrolytic mechanism involved the sequential formation of an intermediary thioester acyl enzyme, with simultaneous release of the alcoholic product, followed by the release of the acidic product and regeneration of the enzyme. Support for this postulated sequential ordered mechanism has been presented recently, including spectrophotometric evidence for an initial burst of *p*-nitrophenol released by the hydrolysis of paraoxon (18, 19). Inhibition studies from the same laboratory, using *para*-hydroxymercuribenzoate (PCMB), were interpreted as demonstrating that a free sulfhydryl group is essential for PON's enzymatic activity (20, 21). PON has only three cysteine residues, and amino acid sequencing of tryptic digests of purified human PON in our laboratory directly demonstrated the presence of two peptides in a disulfide linkage (13, 22). These peptides contained amino acid residues 349–354 and residues 32–45. The third sulfhydryl group, residue 283 (Cys-283), is free, and it is the logical candidate for being the key component of the putative active center of PON, equivalent to Ser-198 in human butyrylcholinesterase (23) or Ser-200 in *Torpedo* acetylcholinesterase (24).

To test the Augustinsson hypothesis, we have expressed recombinant human PONs *in vitro*. Site-directed mutagenesis of the type A (Gln-191) human PON cDNA (C283) was performed to create cDNAs coding for mutant PON enzymes containing either serine (C283S) or alanine (C283A) at residue 283. Thus, it was possible to determine if a cysteine was required at position 283 for PON catalytic activity.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The construction of the human liver (type A) PON cDNA and its preliminary expression *in vitro* have been described elsewhere (13, 25). This PON insert was subcloned into the pGS (Scios Nova, Mountain View, CA) plasmid. Mutant sequences were introduced into the PON cDNA in a PCR-mediated, site-directed mutagenesis protocol similar to that of Chen and Przybyla (26) using mutagenic

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Abbreviations: PON, paraoxonase; PCMB, *para*-hydroxymercuribenzoate.

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oligonucleotide primers to create C283A PON (5′-GGAGACCTTTGGGTTGGAGCCCATCCCAATGGCATGAAAATC-3′) and C283S PON (5′-TTCATGCCATTGGGATGGGATCCAACCCAAAGG-3′). These primers were used in combination with T7 (5′-TAATACGACTCACTATAGGG-3′) or Sp6 (5′-ATTTAGGTGACACTATAG-3′) promoter primers to create partial, mutant PON cDNA amplification products (megaprimers). Megaprimers were extended, using the wild-type pGS/PON cDNA as template, in a second PCR amplification to create full-length mutant PON cDNAs. *Pfu* DNA polymerase (Stratagene) was used in all PCR amplifications. Megaprimers were washed and concentrated in a Centricon-100 (Amicon). Full-length inserts were isolated and washed with a QIAquick spin purification kit (Qiagen, Chatsworth, CA), digested with *Hin*dIII and *Xba* I, and ligated into the pGS plasmid. Expression plasmids were propagated in *Escherichia coli* HB101 cells and purified with the Qiagen plasmid maxi kit. The complete nucleotide sequences of the wild-type and mutant cDNA inserts were determined by the University of Michigan DNA Sequencing Core Facility using automated fluorescent sequencing techniques or manually using dideoxynucleotide chemistry and the *fmol* cycle sequencing kit (Promega). Six artifactual point mutations were identified in the C283 cDNA construct and, consequently, in the mutant constructs (Leu-68, TTA to TTG; Gly-82, GGA to GGT; Lys-137, AAG to AAA; Gly-218, GGA to GGG; Ile-227, ATT to ATC; Ser-260, TCC to TCT). None resulted in an altered amino acid sequence in the expressed enzymes.

Transfection and Expression of Recombinant Enzymes. CHO-K1 cells were maintained in Ham's F-12 medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO_2/95% air and 95% humidity until 24 hr after transfection after which the cells were maintained in Ultraculture (BioWhittaker) medium without any serum supplement but supplemented with 50 μM L-methionine sulfoximine (Sigma) for selection. CHO-K1 cells were transfected with 10 μg of plasmid DNA in a calcium phosphate coprecipitate as described elsewhere (27). Amplification and stable expression of the recombinant enzymes were achieved by using the glutamine synthetase system (27). Recombinant enzymes were collected in medium cultured 4–6 days on confluent cells. Medium was concentrated to 5% of its original volume using a Centricon-30 (Amicon). The rates of enzymatic hydrolysis of phenyl acetate and paraoxon were assayed as described (9).

Determination of Mouse PON Amino Acid Sequence. PON does not show significant sequence homology with any known class of proteins; therefore, the identification of conserved residues critical for PON's stability or catalytic function is currently restricted to those found in known mammalian PONs. In Fig. 1, we have included the amino acid sequence of mouse PON with the known human and rabbit sequences to illustrate the similarities in three mammalian species and to provide a reference from which to study the conserved residues in PON. These sequences also provide a basis for discussion regarding putative canonical sequences for metal binding, which are expected in PON proteins. The mouse amino acid sequence was deduced from the mouse liver PON cDNA sequence. All nucleotide sequences were determined using the *fmol* cycle sequencing kit (Promega). Mouse PON sequences were initially obtained through PCR amplification and subsequent sequencing of genomic mouse DNA from *Mus musculus* with human PON primers PX16 and PX15 (13). Mouse-specific PCR primers were generated and used in combination with λgt11 forward and reverse primers to directly amplify the PON cDNA from a BALB/c liver cDNA library (Clontech). Sequences were verified by sequencing the entire coding region of the cDNA sense and antisense strands.

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Human   MAKLIALTLLGMGLALFRNHQSSYQTRLNALREVQPVELPNCNLVKGIETG
Rabbit  ----T------L-----DGQK--F---F-VH---T------------DN- 50
Mouse   ----L--- V-LV---YK--R---------F---T------------E--

SEDMEILPNGLAFISSGLKYPGIKSFNPNSPGKILLMDLNEEDPTVLELGITGSKFDVS
---L----------A-------M--D-DK----------K--V----S----T--L- 109
A----------T-F-T----------D-S----------KKE-A-S--E-I-NTL-I-

SFNPHGISTFTDEDNAMYLLVVNHPDAKSTVELFKFQEEEKSLLHLKTIRHKLLPNLND
----------|----IV--M------S----------K---------------SV-- 168
----------|----TV------|--SS----V-------R--------T-E---SI--

IVAVGPEHFYGTNDHYFIDPYLRSWEMYLGLAWSYVVYYSPSEVRVVAEGFDFANGINI
----------A------L----K----H------F-T----ND--------------- 227
-A-I---S--A------A-------------S--N------DK-Q------------G-

SPDGKYVYIAELLAHKIHVYEKHANWTLTPLKSLDFNTLVDNISVDPETGDLWVGCHPN
-------------------------------------------V------------ 286
-L-----------------------------V-N-D----------V-----------

GMKIFFYDSENPPASEVLRIQNILTEEPKVTQVYAENGTVLQGSTVASVYKGKLLIGTV
--R--Y--PK----------D--SK-----VA-------------A----M-V--- 345
--R--F--A----G----------S-D--I------------T--------------

FHKALYCEL
---------SQAN
-------D-
```

FIG. 1. Amino acid sequences of human (12, 13), rabbit (12), and mouse PONs. The boxed areas represent possible Ca^{2+}-binding sites (28). Dashes indicate identity with the human enzyme. Cysteine residues are in larger, boldfaced type. Asterisks have been placed above the conserved histidine residues. The first alanine residue is assigned position +1 as it is the first residue in mature serum PON (13). The GenBank accession no. for the mouse cDNA sequence is L40488.

RESULTS

Expression of Wild-Type and Mutant PON in CHO Cells. Fig. 2*A* shows that, although the absolute levels of secreted paraoxonase and arylesterase activity varied significantly between the expressed enzymes, all were 5–79 times greater than the pGS vector mock-transfection control at 1 mM substrate concentrations. Substitution of alanine or serine for Cys-283 did not significantly alter substrate concentration dependence of PON for the hydrolysis of phenyl acetate, as is borne out by the similarity of the apparent K_m (Table 1 and Fig. 2*B*) of each recombinant enzyme for this substrate. The presence of alanine in position 283, however, resulted in an altered K_m for paraoxon hydrolysis as compared to the C283 enzyme ($P < 0.005$). Alterations in residue 283 resulted in enzymes with reduced maximal velocities compared to the C283 enzyme (Table 1 and Fig. 2*B*); however, these estimates represent minimum relative specific activities, as it is not yet possible to distinguish between enzymatically active and inactive PON protein. Given the similarity in the Michaelis constants for the expressed enzymes, it is probable that the observed differences in V_{max} are due, at least in part, to the presence of more inactive

Table 1. Kinetic parameters of the expressed PONs

Enzyme	K_m*, mM		V_{max}*†	
	Phenyl acetate	Paraoxon	Phenyl acetate	Paraoxon
C283	1.16 ± 0.07	0.23 ± 0.03	274.2 ± 20.2	233.7 ± 10.3
C283A	1.16 ± 0.11	0.11 ± 0.03	164.2 ± 8.3	105.1 ± 6.8
C283S	1.04 ± 0.33	0.21 ± 0.03	75.5 ± 15.6	45.1 ± 2.0

*Catalytic constants were obtained by Lineweaver–Burk analyses and represent the mean values ± SD of three independent measurements.
†V_{max} estimates are defined as units (9) per milliliter of expression medium normalized to the amount of expressed mutant protein as a percentage of the expressed C283 protein ± SD (see Fig. 1*C*).

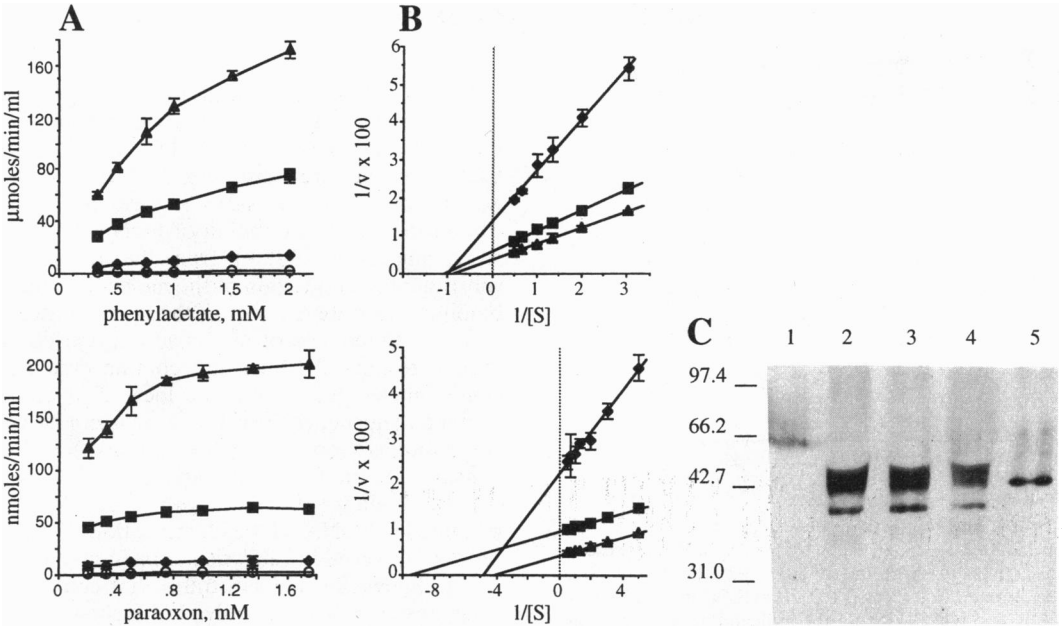

FIG. 2. Paraoxonase and arylesterase activities of wild-type and mutant PONs. (*A*) Concentration dependence of paraoxon and phenyl acetate hydrolysis by expressed PONs. ▲, C283; ■, C283A; ◆, C283S; ○, pGS vector mock transfection. Symbols represent the mean rates of hydrolysis ± SD for three independent experiments. The hydrolysis of phenyl acetate and paraoxon was measured as described (9) except that paraoxon hydrolysis was assayed in 50 mM Tris Cl (pH 7.5) containing 2 mM $CaCl_2$ and 1 M NaCl at 25°C to minimize hydrolysis by albumin. (*B*) Lineweaver–Burk plots of the same data corrected for background activity of the Ultraculture medium and pGS expression vector (2.19 units/ml for paraoxon; 1.65 units/ml for phenyl acetate). (*C*) Western blot analysis of recombinant PONs. Lanes: 1, pGS vector mock transfection; 2, C283 PON; 3, C283A PON; 4, C283S PON; 5, 300 ng of purified human serum PON (10). The migration distances of molecular weight standards are indicated on the left. Expression media from CHO cells transfected with C283, C283A, and C283S constructs as well as the pGS vector without insert were run on a concanavalin A-Sepharose 4B affinity column to partially purify PON. This was done to avoid the effects of the large amounts of bovine serum albumin present in the concentrated Ultraculture medium, which distorted SDS/PAGE gels and the visualization of the PON enzymes, if not removed. The bound enzyme was eluted with 0.5 M methyl-α-D-mannopyranoside in 50 mM Tris Cl, pH 8.0/1 mM $CaCl_2$/5 μM EDTA/0.1% deoxycholate. The eluate was concentrated with a Centriprep-30, diluted in 50 mM Tris Cl, pH 8.0/1 mM $CaCl_2$/5 μM EDTA/20% (vol/vol) glycerol, and reconcentrated. The concentrated preparations were assayed for arylesterase activity and loaded onto a 12% polyacrylamide gel. SDS/PAGE, protein blotting, and immunoblotting were performed as described (27). The enzyme–antibody complex was visualized with goat anti-rabbit IgG conjugated to alkaline phosphatase. The relative amounts of expressed PON protein were estimated through quantitation of Western blots as described elsewhere (29). Rabbit antiserum to purified type AB human PON was kindly provided by Mahamad Navab (University of California, Los Angeles).

enzyme in the C283S and C283A preparations. The fact that recombinant PON retains both arylesterase and paraoxonase activities when expressed in a heterologous cell line refutes earlier claims that serum arylesterase and paraoxonase were distinct enzymes (30, 31).

Western Blot Analysis of Recombinant Enzymes. Four isoforms of purified rabbit PON can be identified with SDS/PAGE analysis (32). The existence of distinctive subpopulations of PON in mammalian sera is further indicated by the presence of three bands in Western blot analyses of recombinant human PON, presented in Fig. 2*C*, and human serum PON, presented elsewhere (33). The persistence of these bands in the presence of SDS, under reducing conditions, suggests that they represent distinct PON glycoforms rather than different oxidative states or conformations of the enzyme (34). Similar analyses using a monoclonal antibody have only detected a single PON band (35). This antibody may have avidity for a specific carbohydrate moiety, and polyclonal antibodies may be required to detect all forms of serum PON. The purified enzyme also shows only one band, and it is possible that our present purification scheme selects for a specific PON subtype.

Titration of Free Sulfhydryl Groups in Purified Human and Rabbit Serum PON and Inactivation of Recombinant PON by PCMB. Quantitative titration of the purified human and rabbit PON enzymes has demonstrated the presence of a single free sulfhydryl group in the native state and three titratable sulfhydryl groups after reduction (Table 2). The C283 recombinant enzyme showed the expected pattern of inhibition by

Table 2. Determination of the number of free cysteine residues in purified rabbit and type A human PONs

Sample	ΔA_{250} ± SE* × 10^3	Free -SH per mole
Reduced glutathione	8.14 ± 0.07	1.00
Native human PON	8.42 ± 0.36	1.03
Reduced human PON	28.86 ± 3.06	3.55
Native rabbit PON	8.21 ± 0.81	1.01
Reduced rabbit PON	24.68 ± 0.74	3.03

Free sulfhydryl (-SH) groups in purified human (type A) and rabbit PONs were titrated with PCMB (Sigma) as described (50). Prior to analyses, the enzyme preparations were extensively washed in 50 mM phosphate buffer (pH 7.0) by Centricon ultrafiltration. The final concentration of the enzymes was 3.0–5.0 mg/ml. Protein concentrations were determined using the Pierce BCA kit with bovine serum albumin as standard. Molarity estimates of the protein solutions were made using a molecular mass of 39.65 kDa. This estimate is of the nonglycosylated protein, because the BCA method does not detect carbohydrates. Aliquots of PON (10 μl) were added sequentially to 1 ml of 70–90 mM PCMB, and the absorbance at 250 nm was recorded after each addition. The change in absorption correlating with the molarity of PON in solution was estimated by the slope of the regression line generated with these data. PON was included in blanks to correct for the absorption of the protein at this wavelength. Reduced glutathione was used as the standard. PON was reduced by preincubating for 1 hr with 5% 2-mercaptoethanol followed by Centricon ultrafiltration to remove unoxidized reductant. To prevent reoxidation of the reduced enzymes, all buffers were aerated with nitrogen for at least 1 hr.

*Standard error of regression.

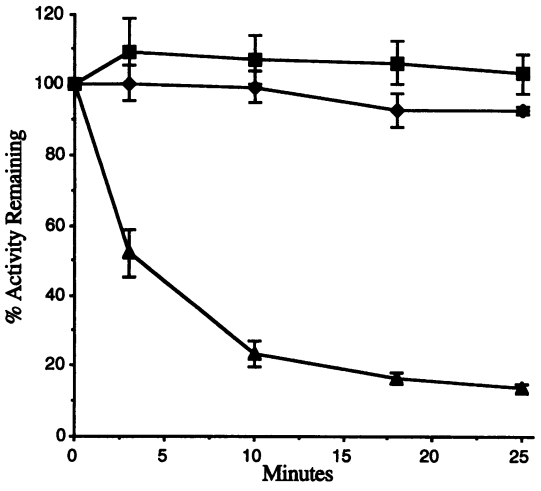

FIG. 3. Time course for the inactivation of expressed recombinant PONs C283 (▲), C283A (■), C283S (◆) by PCMB. Symbols represent the mean residual activity of three independent experiments ± SD. Samples were preincubated in 0.11 mM PCMB at 37°C in 50 mM Tris Cl, pH 8.0/1 mM $CaCl_2$/5 μM EDTA for the indicated times. Residual arylesterase activity was measured with a final concentration of 1 mM phenyl acetate as substrate at 37°C. The reaction was initiated by the dilution of a 10× phenyl acetate stock solution into the incubation mix and measured spectrophotometrically as described (9).

PCMB, whereas the C283S and C283A mutants were completely resistant to inactivation (Fig. 3).

Homology of Mammalian PONs. The deduced amino acid sequence of mouse PON is illustrated in Fig. 1 together with the human and rabbit sequences. The mouse amino acid sequence shows 82% and 81% identity with that of the human and rabbit, respectively. Seventy-six percent of the residues are identical in the sequences of all three species. Cys-41, Cys-352, and the putative active-center nucleophile Cys-283 are conserved in all PON proteins. Three potential calcium-binding loops similar to those of the troponin C superfamily (28) are present in the three mammalian PONs (Asn-77–Asp-88; Asp-121–Asn-132; Asp-262–Asp-273) as are 10 histidine residues (His-114, His-133, His-154, His-160, His-183, His-242, His-245, His-250, His-284, and His-347), which are potential metal ligands or general bases.

DISCUSSION

The model of the PON active center proposed by Augustinsson was based on the sequential catalytic mechanism of the serine esterases, with cysteine as the active-center nucleophile. The phosphoryl-thioester intermediate was predicted to be more susceptible to nucleophilic attack by water to regenerate the enzyme and thus confer the ability to hydrolyze organophosphates catalytically. Subsequent crystallographic studies on the active centers of papain and trypsin revealed that the active-center triads of the cysteine and serine proteases were virtually superimposable, although little homology was found in their overall amino acid sequences (36). Nonetheless, the results of mutagenesis experiments have shown that the replacement of serine by cysteine in the active-center triad of serine esterases (37, 38) or serine proteases (39) results in a significantly less efficient or inactive enzyme. The Augustinsson model implies that the C283S mutant described in this study would retain its ability to hydrolyze phenyl acetate but be inactivated through stoichiometric phosphorylation through the suicide hydrolysis of paraoxon. This model would further predict that the C283A mutant would have no enzymatic activity, as was observed when Ser-203 of human acetylcholinesterase was replaced with alanine (38). The retention of PON activity by the C283A and C283S mutants requires that the Augustinsson model of catalysis, with Cys-283 as the active-center nucleophile, be abandoned.

Recently, Kelso and coworkers (40) suggested that the free cysteine is residue 352. Our finding that the wild-type recombinant PON is inactivated by PCMB, while the C283A and C283S enzymes are resistant to PCMB inhibition, leaves little doubt that residue Cys-283 is free in the native enzyme. Therefore, PCMB inhibition of PON activity must be due to steric hindrance resulting from the introduction of a large substituent near a region of the molecule critical for substrate binding, substrate transfer to the active center, Ca^{2+} binding, or the maintenance of an active enzyme conformation. Although residue 283 need not contain cysteine as an active-center nucleophile, it may be located in or near the active center and influence the catalytic behavior of the enzyme. This conclusion is supported by the finding of an altered Michaelis constant for the C283A mutant.

No crystallographic data are available for the structure of mammalian PON, and the identification of specific amino acid residues involved in catalysis has been impeded because there are no known suicide substrates that could be used to label these residues. Additionally, PON shows no significant sequence homology with any described family of proteins. Therefore, it is not presently possible to predict its catalytic mechanism from that of closely related proteins. PON may, nonetheless, show structural or mechanistic homologies with other enzymes as a result of convergent evolution. For any of these models to be credible, the amino acids requisite for activity should be present in all active PON enzymes. Human and rabbit PONs appear to have two distinct Ca^{2+}-binding sites, one required for stability and one required for catalytic activity, with differing affinities (41). PON's Ca^{2+} dependency suggests a model of metal-catalyzed hydrolysis such as that proposed for phospholipase A_2 in which Ca^{2+} is thought to act as an electrophilic catalyst (42). It is also consistent with studies for the zinc-dependent phosphotriesterase from *Pseudomonas diminuta*, which is known to be an efficient paraoxonase (43). In the phospholipase A_2 and phosphotriesterase models, the active-center metal serves to coordinate a carbonyl or phosphoryl oxygen, respectively, and orients the substrate for an in-line nucleophilic attack by hydroxyl ion generated with a histidine imidazole acting as a general base (42, 44–46). The bacterial phosphotriesterase serves as a particularly appropriate model with two zinc ions coordinating with six histidine residues to form a binuclear metal center with catalytic and structural functions (45). Consistent with this model, 10 histidine residues are conserved in rabbit, human, and mouse PONs (Fig. 1) with residues His-242, His-245, and His-284 positioned to create a metal-binding active-site configuration found not only in the *Pseudomonas* phosphotriesterase but in numerous enzymes that utilize divalent cations in catalysis (47–49). This model would place Cys-283 in the PON active center and provide an explanation for the ability of PCMB to inactivate PON. Additionally, PON activity increases under basic conditions (8), which may be due to the ionization of a nucleophilic, metal-bound water molecule or the deprotonization of imidazole side chains involved in metal binding or general base catalysis. It is important to note, however, that mammalian PONs may represent a class of hydrolases with a different catalytic mechanism.

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