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Review

3-D STRUCTURE OF SERUM PARAOXONASE 1 SHEDS LIGHT ON ITS ACTIVITY, STABILITY, SOLUBILITY AND CRYSTALLIZABILITY*

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Serum paraoxonases (PONs) exhibit a wide range of physiologically important hydrolytic activities, including drug metabolism and detoxification of nerve gases. PON1 and PON3 reside on high-density lipoprotein (HDL) (the "good cholesterol"), and are involved in the alleviation of atherosclerosis. Members of the PON family have been identified not only in mammals and other vertebrates, but also in invertebrates. We earlier described the first crystal structure of a PON family member, a directly-evolved variant of PON1, at 2.2 Å resolution. PON1 is a 6-bladed beta-propeller with a unique active-site lid which is also involved in binding to HDL. The 3-D structure, taken together with directed evolution studies, permitted analysis of mutations which enhanced the stability, solubility and crystallizability of this PON1 variant. The structure permits a detailed description of PON1's active site and suggests possible mechanisms for its catalytic activity on certain substrates.

KEY WORDS: *catalytic mechanism, crystal-forming variants, directed evolution*

Paraoxonase 1 (PON1) is a mammalian enzyme that catalyzes the hydrolysis and, thereby, the inactivation of various organophosphates (OPs), including the nerve agents sarin and soman (1). In recent years it has become apparent that PON1 and its two known Q and R isoforms also play important roles in drug metabolism and in alleviation of atherosclerosis (1, 2). PON1 is the best studied member of the family of mammalian enzymes that includes PON2 and PON3, both of which share ~60 % sequence homology with PON1. PON1 and PON3 are associated with the cholesterol-carrying particles, high-density lipoprotein (HDL) (the "good cholesterol"), whereas PON2 is found in many tissues. Polymorphism of the PON1 gene affects the blood levels of PON1 and its catalytic efficacy, and these factors have a major impact on the individual's susceptibility to atherosclerosis, as well as to pollutants and insecticides (1). Knockout mice, totally lacking PON1, are highly susceptible both to atherosclerosis and to OP poisoning (3). *In vitro* assays show that PON1 and PON3 inhibit lipid oxidation in low-density lipoprotein (LDL) ("bad cholesterol"), thus reducing the levels of oxidized lipids that are involved in initiation and progression of atherosclerosis (4, 5).

The name paraoxonase is purely historical, since the PON family is a hydrolase family with one of the broadest specificities known. PON1 efficiently

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hydrolyzes several synthetic ester substrates, whilst PON2 and PON3 exhibit high lactonase activity. However, the paraoxonase activity of PON1 is rather weak, and both PON2 and PON3 exhibit almost no such activity (1). A variety of physiological roles have been proposed for PONs, including phospholipase A2 action in the hydrolysis of platelet-activating factor (6) as well as of oxidized lipids (7), and hydrolysis and inactivation of homocysteine thiolactone - a known risk factor for atherosclerotic vascular disease (8). The anti-atherosclerotic activity of the PONs is intimately linked to their localization on HDL particles. HDL has two key roles: mediation of cholesterol efflux, e.g. from macrophage foam cells in atherosclerotic lesions, and limitation of lipid oxidation in LDL (9). PONs have been implicated in both (2, 10, 11). It has been suggested that the hydrophobic N-terminus of PON1 mediates its anchoring to HDL (12), but the precise mode of binding of PONs to HDL, as well as of other HDL-associated proteins, is still unknown.

We recently described the directed evolution of PON1 and PON3 variants that are expressed in a soluble and active form in *E. coli*, and exhibit enzymatic properties virtually identical to those reported for PONs purified from sera (13). We also reported the crystal structure of a recombinant PON1 variant derived from rabbit PON1 that is very similar in sequence to human PON1 (14). By combining directed evolution, site-directed mutagenesis and kinetic studies, we analyze the factors which contribute to the variant's enhanced stability and crystallizability, and provide a description of the overall architecture of PON1 and of some of its catalytic mechanisms.

METHODS

Structure determination, directed evolution and enzyme kinetics have been described by Harel et al. (14). Coordinates and structure factors were deposited in the PDB with entry code 1v04.

RESULTS

Crystallization and structure determination

Previous attempts to determine the structure of PON1 relied on limited amounts of serum-purified proteins, and led to the crystallization of a protein that was co-purified with it (15). Human PON1 is rather unstable, and tends to aggregate in the absence of detergents (16). Nor is it amenable to functional expression in bacteria or yeast, and thus to mutagenesis, library selection, and protein engineering. These factors led us to directly evolve PONs for bacterial expression and increased solubility (13). Family shuffling of four PON1 genes (human, rabbit, mouse and rat), followed by screening for esterolytic activity, led to recombinant PON1 variants (rePON1s) that are expressed well in *E. coli*. These variants diverged from the wild-type (wt) rabbit PON1 by 14-32 amino acids contributed by the other three PON1 genes. The rePON1 variants exhibited enzymatic properties essentially identical to those of the wt PON1 (13), and similar biological activities in inhibiting LDL oxidation and mediating cholesterol efflux from macrophages (17, 18). Variants from the first round of evolution displayed a tendency to aggregate, and none could be crystallized. The second-generation variants, obtained by shuffling of the first-generation variants and screening for highest expression levels, did not aggregate, and one (G2E6) yielded stable well-diffracting crystals.

RePON1-G2E6 exhibits 91 % homology to the wt rabbit PON1 (Figure 1), with the majority of variations deriving from human, mouse, or rat wt PON1. Rabbit

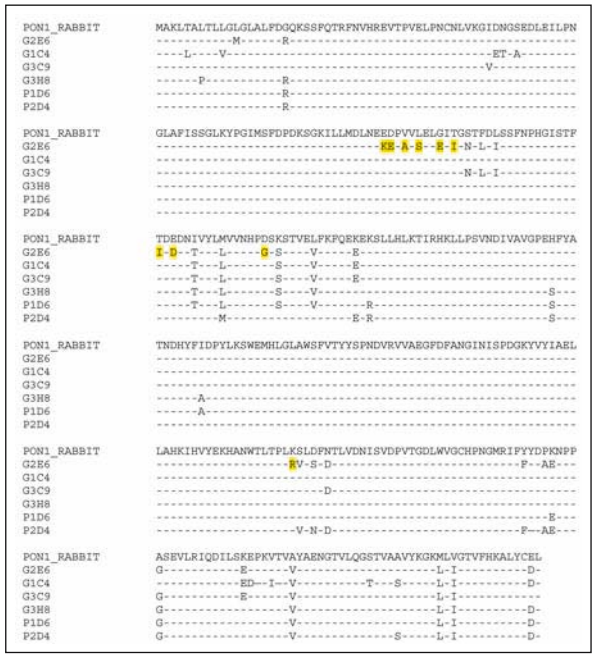

Figure 1 *Sequence alignment of rabbit PON1 with six variants for which crystallization trials were performed. G2E6 is the variant which produced the crystals whose X-ray structure was solved. For each sequence, only the residues that differ from the sequence of rabbit PON1 are shown. Mutations unique to variant G2E6 are highlighted in yellow.*

and human PON1s are also highly homologous in sequence (86 %) and function (19). Sequence variations between rePON1-G2E6 and rabbit and human PON1 are in regions that do not affect their active sites.

The refined 2.2 Å crystal structure of rePON1 (R-factor 18.5 %; R-free 21.7 %) contains one molecule per asymmetric unit. It was solved by single isomorphous replacement anomalous scattering (SIRAS) from data collected on crystals of the native protein and of the selenomethionine (SeMet) protein at 2.6 Å resolution. The structure shows all residues except N-terminal residues 1-15 and a surface loop 72-79. Two calcium atoms, a phosphate ion, and 115 water molecules are also seen.

Rabbit PON1 vs. rePON1 G2E6

Figure 2 shows G2E6, whose X-ray structure shows 31 of the 32 residues (the mutation in residue #12 is in the N-terminus region which has no electron density) that are mutated relative to the rabbit PON1 sequence. Twenty-two of these differences are on the surface of the molecule, and nine are in the core. Nine of the inner core mutations are from one hydrophobic residue to another. It has been suggested that the enhanced stability of G2E6 is due to three mutated residues which are common to all the variants for which crystallization experiments were performed (see Figure 2). These are Met341Leu and Val343Ile, both hydrophobic core residues that pack against each other, and the neighbouring Ala320Val. Roodveldt et al. (20) have suggested that these mutations increase the stability of the calcium-free apo-form of PON1.

Detergent-solubilized PON1 forms dimers and higher oligomers (16), but there is only one molecule per asymmetric unit of the crystal, and very few contacts between symmetry-related molecules. It is possible that crystallization favours a monomeric form.

The catalytic mechanism

The upper of the two calcium ions seen in the crystal structure, Ca-1, lies at the bottom of the active-site cavity, together with a phosphate ion coming from the mother liquor (14). One of this phosphate's oxygens is only 2.2 Å from Ca-1. This phosphate ion may be bound in a mode similar to the intermediates in the hydrolytic reactions catalyzed by PON, with the oxygen adjacent to Ca-1 mimicking the oxyanionic moiety of those intermediates stabilized by the positively-

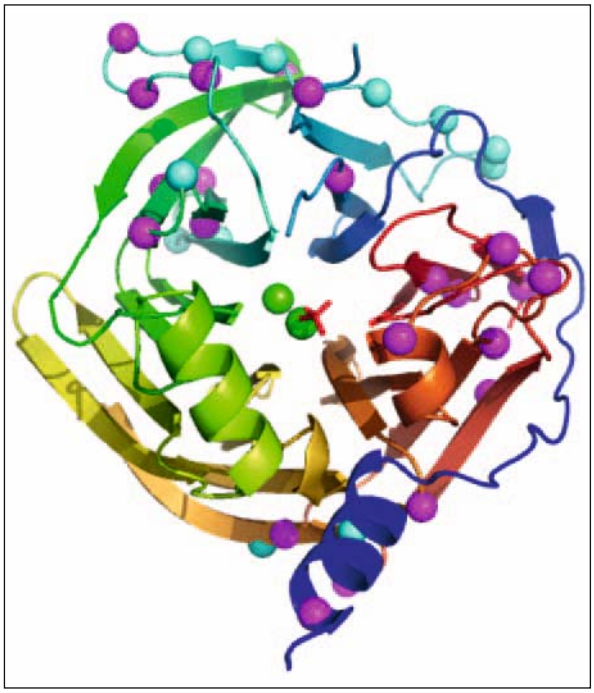

Figure 2 *Cartoon image of the 6-bladed beta-propeller structure of the recombinant PON1 variant G6E2. The variant's mutated residues, relative to wild type rabbit PON1, are shown as magenta and cyan balls, the cyan balls indicating unique mutations in G2E6 not shared by the 5 other variants of PON1 that failed to crystallize. The two calcium ions are shown as green balls, and the phosphate ion as a red stick model. Two of the blades, 3 (green) and 4 (yellow), are mutation-free.*

charged calcium ion. This type of "oxyanion hole" is seen in secreted phospholipase A2 (21), and has also been suggested for diisopropylfluorophosphatase (22), whose overall structure closely resembles that of PON1. Two other phosphate oxygens may be mimicking the attacking hydroxyl ion and the oxygen of the alkoxy or phenoxy leaving groups of ester and lactone substrates.

To help elucidate PON1's mechanism, we determined its catalytic pH-rate profile. The pH-dependence observed may be ascribed to a histidine imidazole involved in a base-catalyzed, rate-determining step. In hydrolytic enzymes, histidine often serves as a base, deprotonating a water molecule, and thus generating the attacking hydroxide ion that produces hydrolysis.

We identified a His-His dyad near both Ca-1 and the phosphate ion (Figure 3). We hypothesize that His115 (the closer nitrogen of which is only 4.1 Å from Ca-1) acts as a general base to deprotonate a single water molecule, thus generating the attacking hydroxide, while His134 acts in a proton shuttle mechanism to increase His115's basicity. Interestingly, His115 adopts

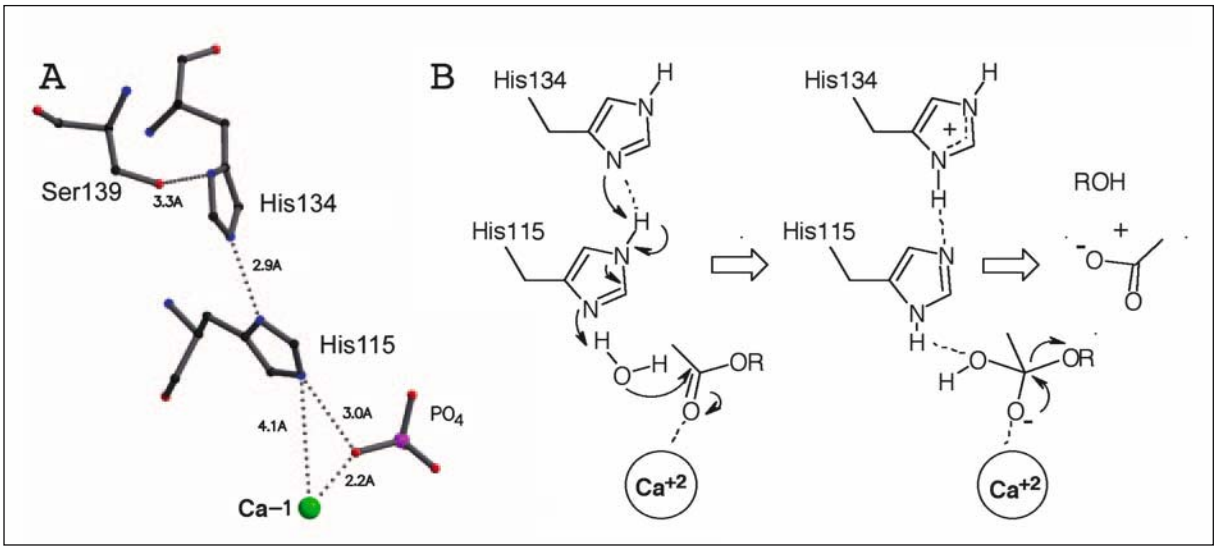

Figure 3 *The postulated catalytic site and mechanism of PON1. A) The catalytic site: the upper calcium atom (Ca-1), the phosphate ion at the bottom of the active site, and the postulated His-dyad; B) Schematic representation of the proposed mechanism of action of PON1 on ester substrates such as phenyl and 2-naphthylacetate. The first step involves deprotonation of a water molecule by the His dyad to generate a hydroxide anion which attacks the ester carbonyl, producing an oxyanionic tetrahedral intermediate. This intermediate breaks down (second step) to an acetate ion and either phenol or 2-naphthol.*

distorted dihedral angles - a phenomenon observed in catalytic residues of many enzymes. In support of the postulated mechanism we investigated His115Gln, His115Ala, and His115Trp mutations, all of which produce a dramatic decrease in both arylesterase and lactonase activity of PON1, and the His134Gln mutation, that resulted in a milder, yet significant, decrease. Interestingly, the paraoxonase activity of the PON1 variant was not affected by any of these His mutations (18).

DISCUSSION

This study complements earlier examples of the application of directed evolution for protein crystallization (23). Directed evolution was also applied to identify PON1's active site, and provided key insights as to how the substrate selectivity of the PON family members evolved in nature. Two thirds of the mutations in the G62E mutant are found on the surface of the molecule. The alignment of all the PON1 variants that were subjected to crystallization trials (Figure 1) show that the crystallized variant G2E6, which was crystallized successfully, has about twice as many mutations as those which could not be crystallized. Thus, it bears 32 mutations vs. 13-19 mutations for the 3 variants which did not crystallize. Ten of these mutations are unique to the G2E6 variant. They are all located on the surface of PON1, yet they do not make contacts with symmetry-related molecules in the crystal. This work shows the advantage of directed evolution in generating a range of variants that are very similar, of which only some might readily form crystals. The mutations in the G2E6 variant are spread throughout most of the molecule except for two blades of the six-bladed beta-propeller, blades 3 and 4, neither of which bears a mutation (see Fig. 2). Detergent-solubilized PON1 forms dimers and higher oligomers (16), although the crystal structure contains only one monomer in the asymmetric unit. It is possible that the mutation-free surface is the site of dimerization of the PON1, being in this respect similar to the highly soluble bacterial phosphotriesterase variants which contain only 3 point mutations, which are all distal from the dimer surface (24); in this latter case the dimer surface is also mutation-free.

The crystal structure unravels both the overall fold of the PON family and the details of PON1's structure. It permits postulation of the catalytic mechanism of the esterase and lactonase functions of PONs. The mutagenesis data (18) indicate that these two activities of PON1 are catalyzed by a His115-His134 dyad, while the paraoxonase activity is not affected by the dyad mutations and, hence, must be catalyzed by different residues.

The directed evolution results demonstrate the remarkable evolvability of this enzyme family, and its

impact on obtaining active, stable, and soluble protein variants which are amenable to crystallization and to subsequent 3-D structure determination.

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REFERENCES

1. Draganov DI, La Du BN. Pharmacogenetics of paraoxonases: a brief review. Naunyn-Schmiedeberg's Arch Pharmacol 2004;369:78-88.
2. Lusis AJ. Atherosclerosis. Nature 2000;407:233-41.
3. Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature 1998;394:284-7.
4. Mackness MI, Arrol S, Durrington PN. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. Fed Eur Biochem Soc Lett 1991;286:152-4.
5. Reddy ST, Wadleigh DJ, Grijalva V, Ng C, Hama S, Gangopadhyay A, Shih DM, Lusis AJ, Navab M, Fogelman AM. Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. Arterioscler Thromb Vasc Biol 2001;21:542-7.
6. Rodrigo L, Mackness B, Durrington PN, Hernandez A, Mackness MI. Hydrolysis of platelet-activating factor by human serum paraoxonase. Biochem J 2001;354:1-7.
7. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor. J Biol Chem 2001;276:24473-81.
8. Jakubowski H. Calcium-dependent human serum homocysteine thiolactone hydrolase. A protective mechanism against protein N-homocysteinylation. J Biol Chem 2000;275:3957-62.
9. Lund-Katz S, Liu L, Thuahnai ST, Phillips MC. High density lipoprotein structure. Front Biosci 2003;8: d1044-54.
10. Fuhrman B, Volkova N, Aviram M. Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized low-density lipoprotein in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase. Atherosclerosis 2002;161:307-16.
11. Rozenberg O, Shih DM, Aviram M. Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: possible role for its phospholipase-A2-like activity and lysophosphatidylcholine formation. Arterioscler Thromb Vasc Biol 2003;23:461-7.
12. Sorenson RC, Bisgaier CL, Aviram M, Hsu C, Billecke S, La Du BN. Human serum Paraoxonase/Arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: apolipoprotein A-I stabilizes activity. Arterioscler Thromb Vasc Biol 1999;19:2214-25.
13. Aharoni A, Gaidukov L, Yagur S, Toker L, Silman I, Tawfik DS. Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. Proc Natl Acad Sci USA 2004;101:482-7.
14. Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, Dvir H, Ravelli RB, McCarthy A, Toker L, Silman I, Sussman JL, Tawfik DS. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat Struct Mol Biol 2004;11:412-9.
15. Morales R, Berna A, Carpentier P, Contreras-Martel C, Renault F, Nicodeme M, Chesne-Seck ML, Bernier F, Dupuy J, Schaeffer C, Diemer H, Van-Dorsselaer A, Fontecilla-Camps JC, Masson P, Rochu D, Chabriere E. Discovery and crystallographic structure of human apolipoprotein. Ann Pharm 2000;65:98-107.
16. Josse D, Ebel C, Stroebel D, Fontaine A, Borges F, Echalier A, Baud D, Renault F, Le Maire M, Chabriere E, Masson P. Oligomeric states of the detergent-solubilized human serum paraoxonase (PON1). J Biol Chem 2002;277:33386-97.

17. Rosenblat M, Gaidukov L, Khersonsky O, Vaya J, Oren R, Tawfik DS, Aviram M. The catalytic His dyad of High Density Lipoprotein-associated serum paraoxonase-I (PON1) is essential for PON1-mediated inhibition of Low Density Lipoprotein oxidation and stimulation of macrophage cholesterol efflux. J Biol Chem 2006;281:7657-66.
18. Khersonsky O, Tawfik DS. The histidine-115 histidine-134 dyad mediates the lactonase activity of mammalian serum paraoxonases. J Biol Chem 2006;281:7649-56.
19. Kuo CL, La Du BN. Comparison of purified human and rabbit serum paraoxonases. Drug Metab Dispos 1995;23:935-44.
20. Roodveldt C, Aharoni A, Tawfik DS. Directed evolution of proteins for heterologous expression and stability. Curr Opin Struct Biol 2005;15:50-6.
21. Sekar K, Yu BZ, Rogers J, Lutton J, Liu X, Chen X, Tsai MD, Jain MK, Sundaralingam M. Phospholipase A2 engineering. Structural and functional roles of the highly conserved active site residue aspartate-99. Biochemistry 1997;36:3104-14.
22. Scharff EI, Koepke J, Fritzsch G, Lucke C, Ruterjans H. Crystal structure of diisopropylfluorophosphatase from *Loligo vulgaris*. Structure (Camb) 2001;9:493-502.
23. Waldo GS. Genetic screens and directed evolution for protein solubility. Curr Opin Chem Biol 2003;7:33-8.
24. Roodveldt C, Tawfik DS. Directed evolution of phosphotriesterase from *Pseudomonas diminuta* for heterologous expression in *Escherichia* coli results in stabilization of the metal-free state. Protein Eng Des Sel 2005;18:51-583.

Sažetak

3-D STRUKTURA SERUMSKE PARAOKSONAZE 1 OBJAŠNJAVA NJEZNU AKTIVNOST, STABILNOST, TOPLJIVOST I KRISTALIZACIJU

Serumske paraoksonaze (PONs) imaju široki raspon fiziološki važnih hidrolitičkih aktivnosti uključujući metabolizam lijekova i detoksikaciju nervnih plinova. PON1 i PON3 smještene su na lipoproteinima visoke gustoće (engl. *high-density lipoprotein*; HDL - "dobri kolesterol") i uključene su u ublažavanje ateroskleroze. Članovi skupine PON identificirani su ne samo u sisavaca i drugih kralježnjaka već i kod beskralješnjaka. Prije smo opisali prvu kristalnu strukturu člana PON skupine, direktno razrađenu varijantu PON1 pri rezoluciji 2,2 Å. PON1 je beta-propeler sa šest lopatica s jedinstvenim poklopcem aktivnog mjesta, koji je također uključen u vezanje na HDL. 3-D struktura, gledana zajedno s direktnim razvojnim istraživanjima, omogućila je analizu mutacija koje povećavaju stabilnost, topljivost i kristalizaciju te PON1 varijante. Struktura dopušta detaljan opis aktivnog mjesta PON1 i sugerira moguće mehanizme za njezinu katalitičku aktivnost prema određenim supstratima.

KLJUČNE RIJEČI: *katalitički mehanizam, usmjerena evolucija, varijante koje tvore kristale*

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