Characterization of human paraoxonase 1 variants suggest that His residues at 115 and 134 positions are not always needed for the lactonase/arylesterase activities of the enzyme

Priyanka Bajaj, Rajan K. Tripathy, Geetika Aggarwal, and Abhay H. Pande*

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar (Mohali) 160062, Punjab, India

Received 27 May 2013; Accepted 23 September 2013

DOI: 10.1002/pro.2380

Published online 3 October 2013 proteinscience.org

Abstract: Human paraoxonase 1 (h-PON1) hydrolyzes variety of substrates and the hydrolytic activities of enzyme can be broadly grouped into three categories; arylesterase, phosphotriesterase, and lactonase. Current models of the catalytic mechanism of h-PON1 suggest that catalytic residues H115 and H134 mediate the lactonase and arylesterase activities of the enzyme. H-PON1 is a strong candidate for the development of catalytic bioscavenger for organophosphate poisoning in humans. Recently, Gupta *et al.* (Nat. Chem. Biol. 2011. 7, 120) identified amino acid substitutions that significantly increased the activity of chimeric-PON1 variant (4E9) against some organophosphate nerve agents. In this study we have examined the effect of these (L69G/S111T/H115W/H134R/R192K/F222S/T332S) and other substitutions (H115W/H134R and H115W/H134R/R192K) on the hydrolytic activities of recombinant h-PON1 (rh-PON1) variants. Our results show that the substitutions resulted in a significant increase in the organophosphatase activity of all the three variants of rh-PON1 enzyme while had a variable effect on the lactonase/arylesterase activities. The results suggest that H residues at positions 115 and 134 are not always needed for the lactonase/arylesterase activities of h-PON1 and force a reconsideration of the current model(s) of the catalytic mechanism of h-PON1.

Keywords: recombinant human PON1; site directed mutagenesis; acyl homoserine lactone; organophosphate

Abbreviations: AChE, acetylcholinesterase; AHL, acyl homoserine lactone; ATCh, acetylthiocholine; CWNA, chemical warfare nerve agent; DTNB, dithionitrobenzoic acid; h-PON1, human paraoxonase 1; rh-PON1, recombinant human paraoxonase 1; OP, organophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HTLactone, homocysteinthiolactone.

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Abhay H. Pande; Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, (Mohali) –160 062, Punjab, India. E-mail: apande@niper.ac.in

Introduction

Human paraoxonase 1 (h-PON1) is a ~40 kDa enzyme synthesized predominantly in the liver and secreted into the bloodstream where it is associated with high density lipoprotein particles.¹ The enzyme is capable of hydrolyzing different type of substrates, for example, arylesters, thioesters, phosphotriesters, carbonates, lactones, and thiolactones.^{2–7} Various hydrolytic activities of h-PON1 can be broadly grouped into three categories; arylesterase, phosphotriesterase, and lactonase.^{2–8} Thus, the h-PON1 is a multi-tasking enzyme and the level and the activity of h-PON1 in

individuals have a major role in determining their susceptibility towards various diseases and other conditions.

The native activity of h-PON1 is lactonase, however, the enzyme possesses considerable phosphotriesterase activity.^{4,5,7} The h-PON1 can hydrolyze and inactivate variety of OP-compounds, including certain pesticides and chemical warfare nerve agents (CWNAs) and the protective role of enzyme against OP-poisoning is well established. Animals deficient in PON1 are more sensitive to OP-poisoning and administration of purified exogenous PON1 have been shown to provide protection against OP-poisoning.^{4,5,9–11} In humans the level and the activity of plasma PON1 have a major impact on the individual's susceptibility to OP-poisoning.^{12,13} Thus, h-PON1 is considered as a new generation antidote (catalytic bioscavenger candidate) for the pre-treatment and therapy of OP pesticides and CWNA poisoning in humans.^{14,15}

Number of laboratories in the world are trying to develop variants of h-PON1 possessing enhanced OP-hydrolyzing activity. Recently, Gupta *et al.* identified amino acid substitutions (mutations) in a 4E9 variant of chimeric-PON1 (Chi-PON1) that significantly increased the hydrolytic activity of the variant against some CWNA.¹⁶ Chi-PON1 is a mammalian PON1 evolved by shuffling the genes of rat, mice, rabbit, and human PON1 and differs considerably from h-PON1 in terms of its amino acid sequence as well as its enzymatic activities and other properties.^{15,17–19} It is proposed that Chi-PON1 variants may not be the good catalytic bioscavenger candidates for the development of antidote against OP-poisoning in humans as use of Chi-PON1 variants may lead to immunological and other complications.^{14–16} Thus, it is important to engineer variant(s) of recombinant h-PON1 having enhanced hydrolytic activity towards desired substrate(s) and whose amino acid sequence is as close as possible to the sequence of native h-PON1.

In this study, we have examined the effect of amino acid substitutions identified in 4E9 variant of Chi-PON1¹⁶ on the hydrolytic activities of rh-PON1. The variant, rh-PON1_(7p), containing seven amino acid substitutions (L69G/S111T/H115W/H134R/R192K/F222S/T332S) was generated by site directed mutagenesis and its hydrolytic activities were compared with rh-PON1_(wt). Our result shows that, compared to rh-PON1_(wt), the rh-PON1_(7p) variant possesses significantly increased OP-hydrolyzing activity. However, the rh-PON1_(7p) also exhibited considerable lactonase as well as arylesterase activities. The results suggest that residues H115 and H134 of h-PON1 are not essential for the lactonase/arylesterase activities of the enzyme. However the variant rh-PON1_(7p) contains five additional substitutions other than the substitutions at H115 and H134 and the possibility of the effect of these other five addi-

tional substitutions on the observed effect on the arylesterase and lactonase activities cannot be ruled out. To address this, we have prepared and analyzed the hydrolytic activities of two more variants of rh-PON1_(wt) enzyme; rh-PON1_(2p) which contains H115W/H134R substitutions and rh-PON1_(3p) which contains H115W/H134R/R192K substitutions. Our results indicate that H115-H134, a proposed catalytic dyad for the lactonase/arylesterase activities of PON1,^{8,16,17} is not always needed for the lactonase and arylesterase activities of h-PON1.

Results

Site-directed mutagenesis, expression and purification of rh-PON1 enzymes

The details of the construction of expression plasmid containing gene for rh-PON1_(wt) enzyme are described in our earlier report.[†] In brief, amino acid sequence of native h-PON1 (Gene bank # P27169) was used to design a gene encoding rh-PON1_(wt) enzyme. A number of factors influence the expression of heterologous recombinant proteins, in soluble and active form, in microbial expression system.^{23–25} These include codon biasness, GC content and formation of a stable secondary structure by the mRNA of the target gene, and the presence of a particular “tag” in the recombinant protein.^{23–25} To express rh-PON1 enzyme in soluble and active form in *Escherichia coli*, a gene encoding rh-PON1_(wt) enzyme was designed using amino acid sequence of h-PON1. The gene was interrogated for the presence of rare codons and mRNA secondary structure by using *Visual gene developer.net* and *Vienna mRNA structure prediction* programs. It was observed that due to codon biasness and the formation of stable secondary structure in the mRNA of the designed gene, the expression efficiency in *E. coli* of this form of the gene would be low. Thus the gene was codon optimized in which the codons rarely used in the *E. coli* was replaced with the codons frequently used. The GC content of the gene was also adjusted to be consonant with that in *E. coli* and decreased as low as possible to prevent the formation of a stable secondary structure in its mRNA. The designed gene was custom-synthesized, cloned into pET23a(+) plasmid, and was purchased commercially from GenScript, NJ. This rh-PON1_(wt) enzyme contains 355 amino acids (Met¹-Leu³⁵⁵) of native h-PON1, have L, H, and R residues at positions 55, 115, and 192, respectively, and contain one extra amino acid (E) at position 356 followed by a (His)₆-tag. The pET-23a(+)-rh-PON1_(wt) plasmid was used as a template to

[†]Bajaj P, Aggarwal G, Tripathy RK, Pande AH, Interplay between amino acid residue at positions 115 and 192: H115 is not always needed for the lactonase and arylesterase activities of human paraoxonase 1. (submitted for publication).

Figure 1. Purification of rh-PON1 enzyme. Representative chromatograms showing resolution of proteins on Q-Sepharose column (A), Superdex-200 column (B), and Ni-Sepharose 6 column (C). (—○—) and (—▲—) denotes the absorbance at 280 nm and paraoxonase activity, respectively, of the eluted fractions. Panels D and E are the images of Coomassie stained (4–20%) SDS-PAGE and Western blot showing electrophoretic analysis of the fractions obtained at various stages of a purification experiment. Lane M, protein molecular weight markers; lane 1, *E. coli* cell lysate; lane 2–4 represents fractions obtained after Q-Sepharose chromatography, gel-filtration chromatography, and affinity chromatography, respectively. Monoclonal mouse anti-human PON1 antibodies were used as a primary antibody in developing the blot. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

generate variants. Comparison of the deduced amino acid sequence of rh-PON1 enzymes with native h-PON1 and Chi-PON1 (G3C9 variant) is given in the Supporting information (Fig. S1). At the amino acid level, the rh-PON1_(wt) share ~99.9% similarity with the native h-PON1. The rh-PON1_(7p) differ from the rh-PON1_(wt) in the following seven positions (L69G/S111T/H115W/H134R/R192K/F222S/T332S).

The recombinant proteins were expressed in *E. coli* BL21(DE3) cells and purified to homogeneity by using ion-exchange chromatography followed by gel-filtration and affinity chromatography. Chromatograms showing the resolution of proteins during a typical purification procedure are given in Figure 1(A–C). The purity of proteins at various stages of purifications was monitored by SDS-PAGE and Western blot analysis [Fig. 1(D,E)]. As evident, after affinity chromatography [Fig. 1(D,E) and lane 4] the purified recombinant protein appeared as a single band with the molecular weight of ~40 kDa (>90% pure; 0.5–0.7 mg/L of *E. coli* culture). The fractions containing paraoxonase activity were pooled, concentrated and used in the enzymatic assays. The K_m and k_{cat} values of rh-PON1_(wt) for phenyl acetate were found to be 2.1 mM and 843.6 s^{−1}, respectively, and for paraoxon were 1.2 mM and 0.89 s^{−1}, respectively. These values are very close to the reported K_m and k_{cat} values of native h-PON1,^{2,17,26–31} suggesting that rh-PON1_(wt) described

in this study is similar to h-PON1 in terms of its enzymatic activities.

Comparison of phosphotriesterase (OP-hydrolyzing) activity

Phosphotriesterase activity of rh-PON1_(wt) and rh-PON1_(7p) was compared using two well-known substrates of PON1; paraoxon and DFP. DFP is a non-hazardous structural analogue of the class-G CWNA. Paraoxon-hydrolyzing activity of the enzymes was determined by a direct assay [Fig. 2(A)]. The rh-PON1_(7p) was >20-folds better in hydrolyzing paraoxon substrate compared to rh-PON1_(wt). DFP-hydrolyzing activity of the enzymes was determined by using acetylcholinesterase inhibition assay and the time course of degradation of DFP by rh-PON1 enzymes are given in Figure 2(B,C). The rh-PON1_(wt) was very poor in DFP-hydrolysis ($k_{obs} = 0.00106 \pm 0.0009 \text{ min}^{-1} \mu\text{M}^{-1}$ of enzyme). Compared to rh-PON1_(wt), the variant was found to be ~100-folds better in DFP-hydrolysis ($k_{obs} = 0.100 \pm 0.01 \text{ min}^{-1} \mu\text{M}^{-1}$ of enzyme). This result was expected and is consistent with the observation that identified amino acid substitutions (L69G/S111T/H115W/H134R/F222S/T332S) considerably increases the OP-hydrolyzing activity of Chi-PON1.¹⁶

Figure 2. OP-hydrolyzing activity of rh-PON1 enzymes. Panel A shows the paraoxonase activity of the enzymes. Panel B shows the time course of AChE inhibition data fitted to single-exponential decay curves ($R^2 = 0.98-0.99$). Data taken from the initial part (~50% OP hydrolysis) of the single-exponential decay curves were used to draw linear plots of $\ln$ (% AChE inhibition) versus time and is presented in panel C. Legends: (●—), rh-PON1_(wt) and (▲—), rh-PON1_(7p). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Comparison of arylesterase (phenyl acetate-hydrolyzing) activity

Arylesterase activity of the enzymes was determined by using phenyl acetate as substrate. Comparison of the specific activities of the enzymes suggests that rh-PON1_(wt) was ~1.8-folds better in hydrolyzing phenyl acetate than the rh-PON1_(7p) variant enzyme [Fig. 3(A)].

Comparison of lactone-hydrolyzing (lactonase) activity

Lactone-hydrolyzing activity of the rh-PON1_(wt) and rh-PON1_(7p) enzymes was compared using three different lactone substrates; δ -valerolactone, 3O-C₁₂AHL and HTLactone [Fig. 3(B)]. The specific activity of rh-PON1_(7p) against δ -valerolactone was

not significantly different than that of rh-PON1_(wt). Against, 3O-C₁₂AHL the specific activity of rh-PON1_(7p) was >4-folds better than rh-PON1_(wt). While, the specific activity of both enzymes toward HTLactone was nearly similar [Fig. 3(B)].

Above results clearly show that rh-PON1_(7p) possesses considerable arylesterase and lactonase activities indicating H115 and H134 are not essential for these activities of the enzyme. However, the rh-PON1_(7p) variant also contains five additional substitutions and the possibility of the effect of these five additional substitutions on the arylesterase and lactonase activities cannot be ruled out. To address this, two more variants of rh-PON1_(wt); rh-PON1_(2p) containing H115W/H134R substitutions and rh-PON1_(3p)-containing H115W/H134R/R192K

Figure 3. Arylesterase and lactonase activities of rh-PON1 enzymes. Panel A and B shows the phenyl acetate- and lactone-hydrolyzing activities of the enzymes. Legends: (■—), rh-PON1_(wt) and (■—), rh-PON1_(7p). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4. Hydrolytic activities of rh-PON1_(2p) and rh-PON1_(3p) enzymes. Panel A and B shows the hydrolytic activities of rh-PON1_(2p) and rh-PON1_(3p) enzymes, respectively, toward indicated substrates. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

substitutions were generated by following the procedure described in Materials and Methods. Purified rh-PON1_(2p) and rh-PON1_(3p) enzymes were used to determine their paraoxon-, phenyl acetate-, and lactone-hydrolyzing activities. Results are presented in Figure 4. Phosphotriesterase and ary-lesterase activities of the variants were compared using paraoxon and phenyl acetate substrates, respectively. Compared to rh-PON1_(wt), the rh-PON1_(2p) and rh-PON1_(3p) variants exhibit around 2 and 3 folds increased paraoxon-hydrolyzing activity, respectively [Fig. 2(A) and 4(A,B)]. This result was expected and is consistent with the observation that substitution of H115W in PON1 results in increased OP-hydrolyzing activity of the enzyme (unpublished observation).^{18,36–39} The rh-PON1_(3p) was > 1.4-folds better in hydrolyzing paraoxon substrate compared to rh-PON1_(2p). This result is also consistent with the observation that 192K containing PON1 exhibits increased OP-hydrolyzing activity.^{2–5,40} Comparison of the phenyl acetate-hydrolyzing activity suggests that the activity of rh-PON1_(2p) and rh-PON1_(3p) variants was less compared to rh-PON1_(wt), and the phenyl acetate-hydrolyzing activity of the variants was in the order: rh-PON1_(wt) > rh-PON1_(7p) > rh-PON1_(2p) > rh-PON1_(3p).

Lactone-hydrolyzing (lactonase) activity of the rh-PON1_(2p) and rh-PON1_(3p) enzymes was determined using three different lactone substrates; δ-valerolactone, 3O-C₁₂AHL and HTLactone (Fig. 4). When δ-valerolactone was used as a substrate, rh-PON1_(3p) exhibited less hydrolytic activity as compared to rh-PON1_(wt) while rh-PON1_(2p) was completely inactive. Against 3O-C₁₂AHL, both rh-PON1_(2p) and rh-PON1_(3p) variants were found to be inactive. When HTLactone was used as a substrate both the rh-PON1_(2p) and rh-PON1_(3p) variants showed good hydrolytic activity and the HTLactone-hydrolytic activity of the variants was in the following order: rh-PON1_(2p) > rh-PON1_(wt) ≈

rh-PON1_(7p) ≈ rh-PON1_(3p). It is interesting to note that rh-PON1_(wt) variant containing only H115W substitution also exhibited considerable phenyl acetate- and δ-valerolactone-hydrolyzing activities as compared to the rh-PON1_(wt) (unpublished observation).

Inhibitor sensitivity of rh-PON1 enzymes

Hydrolytic properties of rh-PON1 enzymes were further characterized by monitoring their susceptibility toward inhibitor. Purified enzyme was treated with (5 mM) EDTA and the residual ary-lesterase activity was determined using phenyl acetate substrate (Fig. 5). Treatment of rh-PON1 enzymes with EDTA resulted in a complete inhibition of their phenyl acetate hydrolyzing activity (Fig. 5) indicating that Ca²⁺-ions are absolutely required for the activity of rh-PON1 enzymes. Human PON1 is a Ca²⁺-dependent enzyme and calcium ions acts as an essential cofactor for the PON1 functions and presence of

Figure 5. Inhibitor (EDTA) sensitivity of rh-PON1 enzymes. Arylesterase activity of rh-PON1 enzymes was determined in the presence and the absence of EDTA using 1 mM phenyl acetate as a substrate. Activity of enzymes in the absence of inhibitor was taken as control and was assigned 100%. Bar-1, rh-PON1_(wt) control; bar-2, rh-PON1_(wt) + EDTA; bar-3, rh-PON1_(7p) control; bar-4, rh-PON1_(7p) + EDTA; bar-5, rh-PON1_(2p) control; bar-6, rh-PON1_(2p) + EDTA; bar-7, rh-PON1_(3p) control, and bar-8, rh-PON1_(3p) + EDTA.

EDTA is known to inhibit various activities of the enzyme.^{27,28}

Discussion

Because of its OP-hydrolyzing (phosphotriesterase) activity, h-PON1 is a strong candidate for the development of a new generation antidote (catalytic bioscavenger candidate) for the pre-treatment and therapy of OP pesticides and CWNA poisoning in humans.^{13–15} However, the native h-PON1 does not possess sufficiently high catalytic activity against variety of OP substrates and attempts to engineer variants of h-PON1 exhibiting enhanced OP-hydrolyzing activity are going on in different laboratories. Recently, Gupta *et al.* identified amino acid substitutions that significantly increased the activity of Chi-PON1 variant (4E9) against some G-type nerve agents.¹⁶ However, since Chi-PON1 is considerably different than h-PON1,^{15,17–19} it is proposed that this engineered variant of Chi-PON1 may not be a good catalytic bioscavenger candidates for the development of antidote against OP-poisoning in humans.^{14–16,32} Thus, it is essential to engineer recombinant PON1 whose amino acid is as close as possible to the sequence of h-PON1.

In this study we have examined the effect of amino acid substitutions identified in 4E9 variant of Chi-PON1 on the hydrolytic activities of rh-PON1 variant containing 192K. Our results show that rh-PON1_(7p) exhibit enhanced (phosphotriesterase) activity against paraoxon and DFP substrates. Interestingly, rh-PON1_(7p) also showed considerable lactone-hydrolyzing (lactonase) as well as phenyl acetate-hydrolyzing (arylesterase) activities. The latter observations suggest that substitutions of His residues at positions 115 and 134 had a minor effect on the lactonase and arylesterase activities of h-PON1_(7p). However the rh-PON1_(7p) contained five additional substitutions other than the substitutions at positions 115 and 134 and the possibility of the effect of these other five additional substitutions on the observed effect on the arylesterase and lactonase activities cannot be ruled out. To address this, we have analyzed the hydrolytic activities of rh-PON1_(2p) and rh-PON1_(3p) variants which contain H115W/H134R and H115W/H134R/R192K substitutions, respectively. As expected the rh-PON1_(2p) and rh-PON1_(3p) variants showed increased phosphotriesterase activity; however, the arylesterase activity of these variants was less compare to rh-PON1_(wt). Interestingly, rh-PON1_(2p) and rh-PON1_(3p) variants showed considerable lactonase activity, compare to rh-PON1_(wt), depending on the type of the lactone substrate.

The h-PON1 is known to hydrolyze variety of substrates, however, the molecular details of catalytic mechanisms are not yet clear. Based on the information obtained from the *in silico* analysis and

the enzymatic characterization of h-PON1,^{18,33–36} and from the crystal structures and the enzymatic characterization of Chi-PON1 variants,^{37–39} different mechanisms are proposed to explain various hydrolytic activities of h-PON1.

It is proposed that active site of PON1 contains catalytic dyad formed by H115-H134, which deprotonate a water molecule and generate the attacking hydroxide ion that mediate the hydrolysis of lactones and arylesterase.^{37–39} In rh-PON1_(7p), rh-PON1_(2p), and rh-PON1_(3p) variants H residues at positions 115 and 134 are substituted by W and R, respectively, and these variants exhibited considerable lactonase and arylesterase activities. These results suggest that H115-H134 are not always needed for the lactonase and arylesterase activities of h-PON1. It is proposed that the active site of PON1 is highly versatile and multiple residues in the active site of the enzyme are capable of carrying out the same hydrolytic reaction and in the absence of any particular catalytic amino acid residue, other residue(s) in the active site take over the role of that catalytic amino acid residue.^{38,39} Thus, it seems that in the absence of H115-H134 residues in rh-PON1_(7p) some other residue(s) (or mechanism) mediate the lactonase and arylesterase activities of enzyme.

Materials and Methods

Site directed mutagenesis

The construction of expression plasmid pET23a(+)-rh-PON1_(wt) containing a codon-optimized gene encoding for rh-PON1_(wt) was described earlier. Amino acid sequence of h-PON1 (Gene bank # P27169) was used to design a gene encoding rh-PON1_(wt) enzyme. The designed gene was codon-optimized, custom-synthesized, cloned into pET23a(+) plasmid, and was purchased commercially from GenScript, NJ. This plasmid was used to generate variants rh-PON1_(2p), rh-PON1_(3p) and rh-PON1_(7p) containing (H115W/H134R), (H115W/H134R/R192K), and (L69G/S111T/H115W/H134R/R192K/F222S/T332S) amino acid substitutions, respectively. The variants were generated by site directed mutagenesis using Quick change multi-site directed mutagenesis kit, by following the procedures recommended by the manufacturer. Details of the primers used for the introduction of desired mutations are given in the Supporting information (Table SI). Mutagenized plasmids were amplified in *E. coli* DH5 α cells, purified and the DNA sequences of the variants were confirmed by bi-directional DNA sequencing (Eurofinn, India). The mutagenized plasmids were then transformed separately into *E. coli* BL21DE3 cells and the transformed cells were used for the expression and purification of rh-PON1 enzymes.

Protein expression and purification

Expression of rh-PON1 enzymes in *E. coli* BL21DE3 was done by following the procedure described

earlier.[†] Briefly, *E. coli* BL21(DE3) cells were streaked on a Luria–Bertani (LB)-agar plate containing 50 µg/mL carbenicillin and 1 mM CaCl₂ and incubated overnight at 37°C. A single colony from the plate was used to initiate a seed culture in LB-broth supplemented with 50 µg/mL carbenicillin and 1 mM CaCl₂ and the seed culture was grown at 37°C for ~8 h. Seed culture (1%) was then inoculated into fresh LB-broth supplemented with 50 µg/mL carbenicillin and 1 mM CaCl₂ and the main culture was grown at 30°C till OD₆₀₀ reached 0.4–0.6. The culture was then induced with 0.5 mM IPTG and at this point the growth temperature was lowered to ensure the maximal expression of recombinant enzymes in soluble and active form, and the cells were further allowed to grow at 20°C for 32 h. The cells were then harvested by centrifugation and the cell pellets were re-suspended in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, 0.1% tergitol, pH 8.0 supplemented with 1 mM β-ME, 0.1 mM of protease inhibitor cocktail and 10 µg/mL lysozyme). The cell suspensions were gently stirred at 25°C for 1 h and then subjected to sonication (60% amplitude, 10 pulses of 1 minute each with 1 minute break after each pulse on ice). The sonicated cell suspensions were immediately cooled on ice and treated with DNase (1 µg/mL) for 1 h. The suspensions were then centrifuged (16000×g, 30 min, 4°C) to separate clear cell supernatant (lysate) from the insoluble debris and the lysate containing soluble and active rh-PON1 enzyme was used for purification. All purification steps were performed at 25°C unless stated otherwise and the chromatography procedure was done using AKTA purifier UPC-10 FPLC protein purification system (GE Healthcare Bio-Sciences, Uppsala, Sweden). The cell lysate was loaded onto a 50 mL of Q-Sepharose column pre-equilibrated with buffer A (20 mM Tris-HCl, pH-8.0, 1 mM CaCl₂, 0.05 % Tergitol). After washing the column with 250 mL of same buffer, bound proteins were eluted using increasing concentrations of NaCl (0.1–1 M) in buffer A. Eluted fractions were analyzed for both protein contents (OD₂₈₀) and enzyme activity (using paraoxon as substrate) and the fractions containing active protein were pooled, concentrated and subjected to gel filtration chromatography using Superdex-200 column. The elution of protein on Superdex-200 column was done at a flow rate of 0.5 mL/min and 2.0 mL fractions were collected. Fractions showing good paraoxonase activity were pooled and subjected to affinity chromatography on a Ni-Sepharose 6 column pre-equilibrated with buffer A containing 150 mM NaCl and 20 mM imidazole. After washing the column with the same buffer, the bound protein was specifically eluted using buffer A containing 150 mM NaCl and 150 mM imidazole. The eluted fractions were monitored for both protein content and enzymatic

activity. The active fractions were pooled and dialyzed against buffer A to remove the imidazole. The samples were then concentrated using Amicon concentrator (MWCO 3 kDa) and were stored at 4°C. The purity of the preparations at various stages of the purification process was monitored by SDS-PAGE (4–20%) and Western blot analysis using monoclonal mouse anti-h-PON1 antibody as primary antibody (a kind gift from Dr. Richard W James, University Hospital, Geneva, Switzerland).

Enzyme assays

Direct assays. Paraoxon-, phenyl acetate-, and lactone-hydrolyzing activities of enzymes were determined by direct assays, as described earlier.[†] Briefly, hydrolysis of phenyl acetate and paraoxon was measured in the activity buffer (20 mM Tris-HCl, pH 8.0-containing 1 mM CaCl₂) while hydrolysis of δ-valerolactone and *N*-oxododecanoyal-DL-homoserine lactone (3O-C₁₂AHL) was measured in bicine buffer (2.5 mM bicine, pH 8.3-containing NaCl, 1 mM CaCl₂ and 0.2 mM *m*-cresol purple). Hydrolysis of HTLactone was measured in the activity buffer-containing 0.3 mM DTNB.²¹ Purified enzyme was incubated with desired substrate (1 mM final concentration) and the product formation was monitored at 270 nm, 405 nm, 412 nm, and 577 nm for phenyl acetate, paraoxon, HTLactone, and δ-valerolactone/3O-C₁₂AHL, respectively.^{8,17} In all the assays, appropriate blanks were included to correct for the spontaneous, non-enzymatic hydrolysis of the substrates. The amount of substrate hydrolyzed (*i.e.* the product formed) was calculated by using the following extinction coefficients: 1310 M⁻¹cm⁻¹ for phenyl acetate, 9100 M⁻¹cm⁻¹ for paraoxon, and 7000 M⁻¹cm⁻¹ for HTLactone.²¹ For δ-valerolactone/3O-C₁₂AHL, a standard curve using HCl was prepared with *m*-cresol purple.⁸

Acetylcholinesterase-inhibition (indirect) assay.

DFP-hydrolyzing activity of the enzymes was measured using acetylcholinesterase inhibition assay.²⁰ Briefly, enzyme (2.0 µM final concentration) was aliquoted in the activity buffer-containing 200 µM of DFP and the reaction mixtures were incubated at 25°C for the indicated time period. At specified intervals, aliquots were withdrawn from the reaction mixtures and diluted (20-folds) in 200 µL of PBS, pH 7.5, containing 0.3 mM DTNB and 0.01 U/mL AChE enzyme. After 5 min of incubation, the residual AChE activity was determined by adding 0.5 mM acetylthiocholine iodide (ATCh) substrate. Absorbance changes, as a result of ATCh hydrolysis, were monitored at 412 nm at regular intervals and the slope of the traces of the reaction was used to calculate the percentage AChE inhibition. The DFP hydrolysis kinetic data was fitted to single-exponential decay curve and

the initial rate of DFP hydrolysis (K_{obs} , $\text{min}^{-1} \mu\text{M}^{-1}$ of enzyme) was estimated from the slope of the linear plot of $\ln$ (% residual DFP) versus time, which parallels the measured decrease in $\ln$ (% AChE inhibition) with reaction time. The linear correlation analysis is based on points taken from the initial part (up to 50% DFP hydrolysis) of the experimental traces.²⁰ Substrate-control (in reaction buffer) lacking rh-PON1 enzyme and AChE-control were run in parallel. The kinetic experiments were performed in duplicate.

Inhibitor sensitivity of rh-PON1 enzymes.

Effect of EDTA on the arylesterase activity of rh-PON1 enzymes was determined by monitoring the phenyl acetate-hydrolyzing activity in the presence and the absence of EDTA. Purified rh-PON1 enzymes were separately incubated with 5 mM EDTA (final concentration) for 15 min at 25°C. After incubation, EDTA-treated and untreated enzyme preparations were used to determine the arylesterase activity using 1 mM phenyl acetate as substrate.⁸

Acknowledgments

This work was supported by the research grants to AHP from NIPER, SAS Nagar. Priyanka Bajaj (CSIR-SPM-SRF) and Geetika Aggarwal (CSIR-SRF) are thankful to CSIR, New Delhi for financial support in the form of CSIR Fellowship. The authors are grateful to Prof. Richard W. James (University Hospital, Geneva, Switzerland) for the gift of monoclonal mouse anti-HuPON1 antibody.

Reference of the submitted sequence: The GenBank accession number of the submitted nucleotide sequences of rh-PON1_(wt) and rh-PON1_(7P) is KC 456192 and KC 456196, respectively.

References

1. La Du BN, Aviram M, Billecke S, Navab M, Primo-Parmo S, Sorenson RC, Standiford TJ (1999) On the physiological role(s) of the paraoxonases. *Chem Biol Interact* 119–120:379–388.
2. La Du BN, Eckerson HW (1984) The polymorphic paraoxonase/arylesterase isozymes of human serum. *Fed Proc* 43:2338–2341.
3. Eckerson HW, Wyte CM, La Du BN (1983) The human serum paraoxonase/arylesterase polymorphism. *Am J Hum Genet* 35:1126–1138.
4. Furlong CE, Richter RJ, Li WF, Brophy VH., Carlson C, Rieder M, Nickerson D, Costa LG, Ranchalis J, Lusis AJ (2008) The functional consequences of polymorphisms in the human PON1 gene. The Paraoxonases: their role in disease development and xenobiotic metabolism. The Netherlands: Springer.
5. Costa LG, Cole TB, Jarvik GP, Furlong CE (2003) Functional genomics of the paraoxonase (PON1) polymorphisms: effects on pesticide sensitivity, cardiovascular disease, and drug metabolism. *Annu Rev Med* 54: 371–392.
6. Draganov DI, La Du BN (2004) Pharmacogenetics of paraoxonases: a brief review. *Naunyn-Schmiedeberg Arch Pharmacol* 369:78–88.
7. Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN (2005) Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res* 46: 1239–1248.
8. Khersonsky O, Tawfik DS (2005) Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry* 44:6371–6382.
9. Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM (1998) Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 394:284–287.
10. Costa LG, McDonald BE, Murphy SD, Omenn GS, Richter RJ, Motulsky AG, Furlong CE (1990) Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. *Toxicol Appl Pharmacol* 103:66–76.
11. Li WF, Furlong CE, Costa LG (1995) Paraoxonase protects against chlorpyrifos toxicity in mice. *Toxicol Lett* 76:219–226.
12. Ritcher RJ, Jarvik GP, Furlong CE (2010) Paraoxonase 1 status as a risk factor for disease or exposure. *Adv Exp Med Biol* 660:29–35.
13. Cole TB, Jansen K, Park S, Li WF, Furlong CE, Costa LG (2010) The toxicity of mixtures of specific organophosphate compounds is modulated by paraoxonase 1 status. *Adv Exp Med Biol* 660:47–60.
14. Lenz DE, Yeung D, Smith JR, Sweeney RE, Lumley LA, Cerasoli DM (2007) Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. *Toxicology* 233:31–39.
15. Rochu D, Chabriere E, Masson P (2007) Human paraoxonase: a promising approach for pre-treatment and therapy of organophosphorus poisoning. *Toxicology* 233:47–59.
16. Gupta RD, Goldsmith M, Ashani Y, Simo Y, Mullokandov G, Bar H, Ben-David M, Leader H, Margalit R, Silman I (2011) Directed evolution of hydrolases for prevention of G-type nerve agent intoxication. *Nat Chem Biol* 7:120–125.
17. Aharoni A, Gaidukov L, Yagur S, Toker L, Silman I, Tawfik DS (2004) Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proc Natl Acad Sci USA* 101:482–487.
18. Otto TC, Harsch CK, Yeung DT, Magliery TJ, Cerasoli DM, Lenz DE (2009) Dramatic differences in organophosphorus hydrolase activity between human and chimeric recombinant mammalian paraoxonase-1 enzymes. *Biochemistry* 48:10416–10422.
19. Rochu D, Renault F, Cléry-Barraud C, Chabrière E, Masson P (2007) Stability of highly purified human paraoxonase (PON1): association with human phosphate binding protein (HPBP) is essential for preserving its active conformation(s). *Biochim Biophys Acta* 1774:874–883.
20. Amitai G, Gaidukov L, Adani R, Yishay S, Yacov G, Kushnir M, Teitlboim S, Lindenbaum M, Bel P, Khersonsky O (2006) Enhanced stereoselective hydrolysis of toxic organophosphates by directly evolved variants of mammalian serum paraoxonase. *FEBS J* 273: 1906–1919.

21. Ellman GL, Courtney KD (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:91–96.
22. Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, La Du BN (2000) Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. *Drug Metab Dispos* 28:1335–1342.
23. Graslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, Knapp S, Oppermann U, Arrowsmith C, Hui R, Ming J, dhe-Paganon S, Park HW, Savchenko A, Yee A, Edwards A, Vincentelli R, Cambillau C, Kim R, Kim SH, Rao Z, Shi Y, Terwilliger TC, Kim CY, Hung LW, Waldo GS, Peleg Y, Albeck S, Unger T, Dym O, Prilusky J, Sussman JL, Stevens RC, Lesley SA, Wilson IA, Joachimiak A, Collart F, Dementieva I, Donnelly MI, Eschenfeldt WH, Kim Y, Stols L, Wu R, Zhou M, Burley SK, Emtage JS, Sauder JM, Thompson D, Bain K, Luz J, Gheyi T, Zhang F, Atwell S, Almo SC, Bonanno JB, Fiser A, Swaminathan S, Studier FW, Chance MR, Sali A, Acton TB, Xiao R, Zhao L, Ma LC, Hunt JF, Tong L, Cunningham K, Inouye M, Anderson S, Janjua H, Shastry R, Ho CK, Wang D, Wang H, Jiang M, Montelione GT, Stuart DI, Owens RJ, Daenke S, Schutz A, Heinemann U, Yokoyama S, Bussow K, Gunsalus KC (2008) Protein production and purification. *Nat Methods* 5:135–146.
24. Mirzahoseini H, Mafakheri N, Mohammadi S, Enayati N, Mortazavidehkordi N (2010) Heterologous proteins production in *Escherichia coli*: an investigation on the effect of codon usage and expression host optimization. *Cell J* 12:453–458.
25. Maertens B, Spriestersbach A, von Groll U, Roth U, Kubicek J, Gerrits M, Graf M, Liss M, Daubert D, Wagner R, Schäfer F (2010) Gene optimization mechanisms: A multi-gene study reveals a high success rate of full length human proteins expressed in *Escherichia coli*. *Protein Sci* 19:1312–1326.
26. Camps J, Pujol I, Ballester F, Joven J, Simo JM (2011) Paraoxonases as potential antibiofilm agents: Their relationship with quorum-sensing signals in gram-negative bacteria. *AAntimicrob Agents Chemother* 55:1325–1331.
27. Gan KN, Smolen A, Eckerson HW, La Du BN (1991) Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab Dispos* 19:100–106.
28. Jakubowski H (2000) Calcium-dependent human serum homocysteinethiolactone hydrolase: A protective mechanism against protein N-homocysteinylation. *J Biol Chem* 275:3957–3962.
29. La Du, BN, Adkins S, Kuo CL, Lipsig D (1993) Studies on human serum paraoxonase/arylesterase. *Chem Biol Interact* 87:25–34.
30. Costa LG, McDonald BE, Murphy SD, Omenn GS, Richter RJ, Motulsky AG, Furlong, CE (1990) Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats 1. *Toxicol Appl Pharmacol* 103:66–76.
31. Otto TC, Kasten SA, Kovaleva E, Liu Z, Buchman G, Tolosa M, Davis D, Smith JR, Balcerzak R, Lenz DE (2010) Purification and characterization of functional human paraoxonase-1 expressed in *Trichoplusia* larvae. *Chem Biol Interact* 187:388–392.
32. Sarkar M, Harsch CK, Matic GT, Hoffman K, Norris JR, Otto TC, Lenz DE, Cerasoli DM, Magliery TJ (2012) Solubilization and humanization of paraoxonase-1. *J Lipids* 2012:610937.
33. Blum MM, Chen JC (2010) Structural characterization of the catalytic calcium binding site in diisopropylfluorophosphatase (DFPase) and comparison with related β -propeller enzymes. *Chem Biol Interact* 187: 373–379.
34. Sanan TT, Muthukrishnan S, Beck JM, Tao P, Hayes CJ, Otto TC, Douglas MC, David EL, Christopher MH (2010) Computational modeling of human paraoxonase 1: preparation of protein models, binding studies, and mechanistic insight. *J Phys Org Chem* 23:357–369.
35. Hu X, Jiang X, Lenz DE, Cerasoli DE, Wallqvist A (2009) In silico analyses of substrate interactions with human serum paraoxonase 1. *Proteins* 75:486–498.
36. Yeung DT, Lenz DE, Cerasoli DM (2005) Analysis of active-site amino-acid residues of human serum paraoxonase using competitive substrates. *FEBS J* 272: 2225–2230.
37. Khersonsky O, Tawfik DS (2006) The histidine 115-histidine 134 dyad mediates the lactonase activity of mammalian serum paraoxonases. *J Biol Chem* 281: 7649–7656.
38. Ben-David M, Elias M, Filippi JJ, Duñach E, Silman I, Sussman JL, Tawfik DS (2012) Catalytic versatility and backups in enzyme active sites: the case of serum paraoxonase 1. *J Mol Biol* 418:181–196.
39. Ben-David M, Wiczyk G, Elias M, Silman I, Sussman JL, Tawfik DS (2013) Catalytic metal ion rearrangements underline promiscuity and evolvability of a metalloenzyme. *J Mol Biol* 425:1028–1038.
40. Stevens RC, Suzuki SM, Cole TB, Park SS, Richter RJ, Furlong CE (2008) Engineered recombinant human paraoxonase 1 (rHuPON1) purified from *Escherichia coli* protects against organophosphate poisoning. *Proc Natl Acad Sci USA* 105:12780–12784.