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Research paper

Interplay between amino acid residues at positions 192 and 115 in modulating hydrolytic activities of human paraoxonase 1

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ABSTRACT

Human paraoxonase 1 (h-PON1) is a Ca^{2+}-dependent serum enzyme that catalyzes the hydrolysis of different types of substrates. The crystal structure of h-PON1 is not solved yet and the molecular details of how the enzyme catalyzes different types of reactions are not clear. Literature suggests that the amino acid residues at positions 192 and 115 are important for various hydrolytic activities of h-PON1. It is proposed that catalytic residue H115 (and H134) mediates the lactonase and the arylesterase activities of the enzyme while the amino acid residue at position 192 modulates various other hydrolytic activities of the enzyme. However, the relationship between these two residues in the hydrolytic activities of h-PON1 is not studied in detail. In this study, we have expressed and purified the wild-type recombinant h-PON1 (rh-$PON1_{(wt)}$) and its point mutants differing in the amino acid residues at positions 192 and/or 115 using an *Escherichia coli* expression system. The hydrolytic activities of the purified enzymes were compared using enzymatic assays. Our results, for the first time, show that (a) the presence of a particular amino acid residue at position 192 differentially alters the effect of the H115W substitution, and (b) H115 residue is not always needed for the lactonase and arylesterase activities of the enzyme. The results also suggest that the amino acid residues at position 192 and 115 act in conjunction in modulating the hydrolytic activities of the enzyme.

1. Introduction

Human paraoxonase 1 (h-PON1)[1] is a ~45 kDa, Ca^{2+}-dependent serum enzyme capable of hydrolyzing a variety of substrates [1,2].The precise physiological function(s) of h-PON1 is not known yet, however, the enzyme has been shown to possess anti-inflammatory, anti-oxidative, anti-atherogenic, anti-diabetic and organophosphate (OP)- and quorum sensor-hydrolyzing activities [2–7]. Thus, 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 to various diseases [2,5,7].

The role of glycosylation in the catalytic properties of h-PON1 is not clear but a recombinant enzyme lacking glycosylation was found to be as active as the native enzyme [8]. Although h-PON1 has been shown to exhibit multiple hydrolytic activities, the physiological substrate(s) for the enzyme is not known yet. The enzyme is capable of hydrolyzing different types of substrate molecules, e.g., arylesters, thioesters, phosphotriesters, carbonates, lactones and thiolactones [9–14] and its activities can therefore be broadly grouped into three categories, namely; arylesterase, phosphotriesterase and lactonase activities [9–15]. Recent reports suggest that the catalytic activity of native PON1 is lactonase activity [14,15].

Human PON1 is a polymorphic enzyme and two polymorphisms have been reported in the coding region of the enzyme; one at position 55 (L/M) and the other at position 192 (R/Q) [9–12]. It is observed that the polymorphism at position 55 does not affect the catalytic properties of the enzyme, while presence of Q/R at position 192 significantly modulates the phosphotriesterase and lactonase activities of the enzyme [9–12,16–19]. It is also reported that rabbit PON1 and chimeric-PON1 (Chi-PON1), which has a lysine (K) at position 192, possess considerably higher phosphotriesterase activity than h-PON1 containing R/Q at the same position [18,21,23].

Abbreviations: AChE, acetylcholinesterase; AHL, acyl homoserine lactone; ATCh, acetylthiocholine; CPO, chlorpyrifos oxon; chi-PON1, chimeric-paraoxonase 1; CWNA, chemical warfare nerve agent; DZO, diazoxon; DTNB, dithionitrobenzoic acid; h-PON1, human paraoxonase 1; rh-PON1, recombinant human paraoxonase 1; ONPG, *O*-nitrophenyl β-D-galactopyranoside; OP, organophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HTLactone, homocysteinthiolactone; LDL, low density lipoprotein; Me-DEPCyC, 3-cyano 4-methyl 7-diethyl phosphocoumarin.

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The crystal structure of h-PON1 has not been solved yet and the molecular details of how the enzyme catalyzes different types of reactions are also not clear. However, the crystal structures of G2E6 variant of Chi-PON1, a recombinant PON1 evolved for soluble expression in *Escherichia coli* by shuffling the genes of rat, mice, rabbit and human PON1, has been solved [20]. This variant of Chi-PON1 shares ~85% amino acid sequence similarity with h-PON1. Based on the information obtained from the crystal structures of Chi-PON1, *in silico* analysis and the enzymatic characterization of h-PON1 and Chi-PON1, different mechanisms were proposed to explain the hydrolysis of different substrates by h-PON1 [20–27]. It was proposed that H115 residue played an important role in the lactonase and arylesterase activities and the substitution of H115 with W dramatically decreased these activities of the enzyme [16–25].

It is clear from the above discussion that residues at positions 192 and 115 play an important role in various hydrolytic activities of h-PON1. However, the relationship between the two residues has not been studied in detail. Thus, in this study, mutants of the enzyme containing H115W/Q192, H115W/R192 and H115W/R192K were prepared and characterized.

The expression and purification of recombinant h-PON1 (rh-PON1) in the active form using a standard microbial-expression system has so far been elusive [18,28–29]. In this investigation, we have expressed and purified rh-PON1$_{(wt)}$ and its mutants by using codon-optimized gene and an *E. coli* expression system. The hydrolytic activities of the purified enzymes were compared using enzymatic assays. Our results show that the presence of a particular amino acid residue (K/Q/R) at position 192 differentially alters the hydrolytic activities of H115W substituted rh-PON1. The results suggest that the amino acid residues at positions 192 and 115 act in conjunction in modulating the hydrolytic activities of the enzyme.

2. Materials and methods

2.1. Materials

2-Hydroxyquinoline (2-HQ),5-bromo-4-chloro-3-*O*-indolyl-phosphate-*p*-toluidine salt (BCIP), acetylthiocholine iodide, acetylcholinesterase (from human RBC), alkaline phosphatase-labeled anti-mouse secondary antibody, mouse anti-His primary antibody, arabinose, chloroform, *δ*-valerolactone, gentamycin, *m*-cresol purple, nitro-blue tetrazolium chloride (NBT), *N*-oxodecanoyl-DL-homoserine lactone, *o*-nitrophenyl β-D-galactopyranoside, phenyl acetate, potassium bromide, protease inhibitor cocktail, paraoxon-ethyl, trisodium citrate, and tergitol NP-10 were purchased from Sigma–Aldrich, Bangalore, India. LB media, carbenicillin, and gentamycin were purchased from HiMedia, Mumbai, India. Stratagene's Quikchange site-directed mutagenesis kit was purchased from Agilent Technologies, Gurgaon, India. *E. coli* BL21(DE3), *E. coli* DH5*α* cells and pET23a(+) plasmid were purchased from Novagen, Bangalore, India. Gene JET™ Gel Extraction Kit, T4 DNA ligase, dNTPs, restriction enzymes, nuclease-free water and DNA ladder were obtained from Fermentas, CA, USA. Protein molecular weight markers, and Bradford reagent were purchased from Bio-Rad, Gurgaon, India. Ni-Sepharose 6 columns (5 ml) and HiPrep 16/60 Superdex-200 columns were from GE Healthcare Bio-Sciences Ltd., Uppsala, Sweden. Chlorpyrifos oxon (CAS 5598-15-2; 98% purity) and diazoxon (diazinon-o-analog; CAS 962-58-3; 96% purity) were purchased from Chem. Service, Inc., West Chester, PA, USA. All other reagents used were of analytical grade. Buffers used were prepared in double distilled water.

2.2. Construction of expression plasmid

Construction of expression plasmid pET23a(+) containing the codon-optimized gene encoding rh-PON1$_{(wt)}$ constructs differing in the position of (His)$_6$-tag and their partial characterization are described in Supplemental information (Figs. S1–S3).

2.3. Site-directed mutagenesis

The pET23a(+) plasmid containing a gene for rh-PON1$_{(wt)}$, which contains H and R residue at positions 115 and 192, respectively, and a C-terminus (His)$_6$-tag, was used as a template to generate point mutants in which the amino acid residues at positions 192 and/or 115 were substituted by other residue(s). The mutants were generated by site-directed mutagenesis using Quikchange site-directed mutagenesis kit by following the procedure "recommended by the manufacturer". The primers used for the introduction of mutations of interest were designed using Primer X software (www.bioinformatics.org/primerx/) and are as follows: H115W-f (5′-GTTAGCTCGTTCAACCCGACCGGCATTAGTACGTTTACC-3′), H115W-r (5′-GGTAAACGTACTAATGCCGGTCGGGTTGAACGAGC-TAAC-3′); R192K-f (5′-CTTCTTAGACCCGTATCTGAAAAGCTGGGA-GATGTACCTGG-3′), R192K-r (5′-CCAGGTACATCTCCCAGCTTTTCAG ATACGGGTCTAG-3′) and R192Q-f (5′-CTTCTTAGACCCGTATCTGCA-GAGCTGGGAGATGTACCTGG-3′), R192Q-r (5′-CCAGGTACATATCCC AGCTCTGCAGATACGCCTCTAAGAAG-3′). Mutagenized plasmids were amplified in *E. coli* DH5*α* cells, purified and the DNA sequences of the mutants were confirmed by bi-directional DNA sequencing (Eurofinn, India). The plasmids were then transformed into *E. coli* BL21(DE3) cells and the transformed cells were used for the expression and purification of the recombinant (His)$_6$-tagged enzymes.

2.4. Purification of rh-PON1 mutants

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 Ltd., Uppsala, Sweden). The procedure followed for the expression of recombinant proteins in *E. coli* BL21(DE3) cells and the lysis of cells are described in the Supplemental materials. Clear cell lysates, containing soluble and active rh-PON1 enzymes, were subjected to 60% (w/v) ammonium sulfate precipitation overnight at 4 °C. On the following day the precipitates were collected by centrifugation (16,000 × *g*, 10 min, 4 °C), dissolved in the minimum volume of the buffer A (50 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.1% tergitol, pH 8.0) and were dialyzed against the same buffer at 4 °C. The samples were then centrifuged to remove any insoluble material and the clear supernatants containing the recombinant proteins (rh-PON1 enzymes) were subjected to gel filtration chromatography using Superdex-200 column, pre-equilibrated with buffer A. The elution was done at a flow rate of 0.5 ml/min and 2.0 ml fractions were collected. Fractions showing paraoxonase activity were pooled and subjected to affinity chromatography on a Ni-Sepharose 6 column pre-equilibrated with buffer A containing 10 mM imidazole. After washing the column with the same buffer, the bound protein was specifically eluted using buffer A containing 150 mM imidazole. The eluted fractions were monitored for both the protein content (OD_{280}) and the enzymatic activity (using paraoxon as a substrate). The active fractions were pooled and the pool was dialyzed against buffer A to remove imidazole. The pooled samples were then concentrated using Amicon concentrator (MWCO 3 kDa) and were stored at 4 °C. The purity of the isolated proteins was evaluated by SDS-PAGE [30]. Protein bands on the gel were detected by silver staining.

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