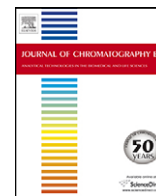




Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb



Integrative analytical approach by capillary electrophoresis and kinetics under high pressure optimized for deciphering intrinsic and extrinsic cofactors that modulate activity and stability of human paraoxonase (PON1)[☆]

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ARTICLE INFO

Article history:

Received 7 July 2009

Accepted 13 November 2009

Available online xxx

Keywords:

Paraoxonase

Human phosphate binding protein

High hydrostatic pressure

Oligomerization

Functional stability

Capillary electrophoresis

ABSTRACT

Paraoxonase (PON1) is working *in vivo* in a particular dynamic environment including HDL particles and associated molecules. To decipher the respective and/or concomitant role of the different cofactors involved in this molecular organization, an approach using multiple experimental techniques based on capillary electrophoresis and classical kinetics or kinetics under high pressure was implemented. The effects of calcium and phosphate as protein or plasma cofactor, of human phosphate binding protein (HPBP) as enzyme chaperone, and of a PON1 inhibitor as an active site stabilizer, on the catalytic activities and functional oligomerization of PON1 were scrutinized. PON1 displays two distinct catalytic behaviors, one against esters and lactones, the other against organophosphorus compounds; its functional states and catalytic activities against these substrates are differently modulated by the molecular environment; PON1 exists under several active multimeric forms; the binding of HPBP amends the size of the oligomeric states and exerts a stabilizing effect on the activities of PON1; PON1 functional properties are modulated by HPBP, calcium and phosphate. This integrative approach using several optimized analytical techniques allowed performing comparison of catalytic properties and oligomeric states of functional PON1 in different enzyme preparations. Relevance of these data to understand *in vivo* physiological PON1 functioning is mandatory.

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1. Introduction

Although there have been significant progress in the past 20 years in medical countermeasures of poisoning by organophosphorus compounds (OPs), classical pharmacological approaches suffer from certain deficiencies. As a naturally occurring enzyme present in plasma, and playing a role in natural defense against OP, human paraoxonase-1 (PON1; EC 3.1.8.1) is the most promising catalytic bioscavenger candidate for pretreatment and therapy of OP poisoning [1,2]. PON1 is an enzyme secreted into the blood where it resides on cholesterol-carrying HDL particles (the “good cholesterol”). The enzyme displays antiatherogenic and detoxification

properties. Because of its implication in such a variety of pathways, PON1 has generated clinical interest over half a century despite lack of consensus on its precise biological role. Compounds that can be hydrolyzed by PON1, e.g., organophosphates, chemical warfare agents, and aromatic esters are human-made chemicals. These non-physiological functions of PON1 are considered as promiscuous activities. The recently described lactonase activity of PON1 [3] is suggested now to be the physiological activity of the enzyme [4].

Thorough characterization of PON1 has long been difficult since the protein was insufficiently purified and highly unstable in solution. Currently, converging data indicate that both activity and stability of PON1 are dramatically dependent on the HDL components. The particular environment of multiple and varying interacting lipids and proteins may explain why study of PON1 in solution was thorny. Thus, the determination of the three-dimensional structure of human PON1 has been a desperate challenge for more than a decade. Thanks to the crystal structure of a recombinant hybrid mammalian PON1 variant (rPON1) obtained by directed evolution, the protein was shown to have a six-bladed β -propeller structure with a unique active site lid [5]. This structure gave new impetus to understand PON1 mechanism and provided

Abbreviations: HPBP, human phosphate binding protein; 2-HQ, 2-hydroxyquinoline; OP, organophosphorus compound; OPH, OP-hydrolase; PON1, paraoxonase 1.

[☆] This paper is part of the special issue “Bioanalysis of organophosphorus toxicants and corresponding antidote”.

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doi:10.1016/j.jchromb.2009.11.027

Please cite this article in press as: F. Renault, et al., J. Chromatogr. B (2009), doi:10.1016/j.jchromb.2009.11.027

a model for PON1 anchoring onto HDL [6,7]. Due to the specific requirements for purified PON1 to be functionally stabilized, the oligomeric state of the human enzyme was very scarcely explored. Indeed, *in vitro* studies on PON1 had to be performed in detergent-containing solutions aimed to mimic the complex and dynamic *in vivo* hydrophobic HDL environment, i.e., an extremely arduous task. A previous work reported globular dimers, trimers and tetramers or a monomer–dimer reversible equilibrium, in nonmicellar or in micellar solutions, respectively [8]. It was finally asserted that the effective stoichiometry of PON1 *in vivo* could not be definitively established in this study.

Our research took an unexpected turn as a novel protein, isolated by co-purification with PON1 from human plasma [9], led to the crystal structure of a protein termed human phosphate binding protein (HPBP) [10,11]. PON1 is a calcium-dependent enzyme and HPBP is a phosphate transporter, and their successful separation is achievable by chromatography on hydroxyapatite (i.e., a sintered form of calcium phosphate) [12]. Because association of PON1 with HPBP was found to be essential for preserving active conformation(s) of the enzyme [13], a rational approach was to re-investigate the behavior of stabilized PON1 oligomeric states in the presence of HPBP, calcium and phosphate. PON1 functions in blood in a very complex and dynamic HDL milieu, which include up to 90 permanently or transiently associated proteins [14]. No clear physiological role was assigned to PON1 which was described as being involved in multiple biological and enzymatic functions. Therefore, an integrative approach, a molecular environment is needed to understand PON1 in action. The time, as a dimension is also needed to understand the dynamic properties of the enzyme.

In the present paper, we describe the examination of the association of human PON1 with HPBP, in an *in vitro* system involving a detergent to surrogate the natural HDL milieu, calcium as the cofactor essential for activity and stability of PON1, and phosphate as the ligand specifically transported by HPBP. An important challenge is to find stable administrable PON1 preparations with enhanced catalytic efficiency. Investigated parameters influencing stability and activity of PON1 are calcium and phosphate as intrinsic protein cofactors or as plasma cofactors, HPBP as a chaperone, and 2-hydroxyquinoline (2-HQ), an inhibitor, as a possible active site stabilizer. The relationships between the composition and the size of multimeric states of PON1 are not well understood, and the effect of HPBP on PON1 catalysis and stability is not clear. We previously showed that PON1 alone or in PON1–HPBP complexes, display at least two distinct oligomeric forms [15]. To determine oligomeric state(s) of functional PON1, and the type of their molecular associations with HPBP, the enzyme functional state(s) were explored using complementary methodologies. This approach included optimized capillary electrophoresis (CE) to follow the behavior of PON1 populated fractions, oligomers distribution, and analysis of activity in the presence of several cofactors, and under perturbing conditions. The effect of pressure on structural stability and activity of different PON1 preparations (PON1 free or in the presence of HPBP, rPON1) recently initiated [16], was expanded by describing the pressure effects on the behavior and the HPBP-dependence of PON1 lactonase, arylesterase and OPH activities. At the same time, the catalytic and structural behaviors of a mammalian rPON1 was compared to that of the natural human enzyme, in order to determine whether this recombinant enzyme can be a convenient model to mimic the physiological PON1 chaperoned by HPBP.

2. Experimental

2.1. Chemicals

DMF, used as the electroosmotic flow (EOF) marker, was from Pierce (Rockford, IL, U.S.A.). 2-Coumaranone (2-Coum) was

obtained from Aldrich (Stainheim, Germany). All other chemicals were of analytical grade and were purchased from Sigma–Aldrich Chimie (L'Isle-d'Abeau, France). Macrosep 30 concentrators were obtained from Pall Life Science (Ann Arbor, MI). All buffer and wash solutions were prepared with Millipore water (18 M Ω cm), filtered through 0.45 μ m filters (Schleicher & Schuell, Dassel, Germany) and degassed before use.

2.2. Enzymes

Human PON1 was purified from pooled plasma (Établissement Français du Sang Rhône-Alpes, Beynost, France) as previously described [12] to obtain either free PON1 or PON1 complexed to HPBP (PON1/HPBP). Briefly, plasma was supplemented with CaCl₂ to a final concentration of 10 mM at 25 °C to induce clotting of fibrin. The filtrate was submitted to pseudo-affinity chromatography on Cibacron Blue 3GA-agarose (type 3000-CL) (Sigma) using 50 mM Tris–HCl buffer, pH 8.0, supplemented with 1 mM CaCl₂ and 3 M NaCl to avoid adsorption of albumin. Elution of hydrophobic proteins, was performed using 0.1% sodium deoxycholate and 0.1% Triton X-100 in the above-noted Tris buffer. PON1-containing fractions were pooled and separated from the other HDL-bound proteins by chromatography on DEAE Sepharose Fast Flow, Q Sepharose Fast Flow or Mono Q (Amersham Biosciences) using 25 mM Tris buffer containing 0.1% Triton X-100 as starting buffer with a NaCl gradient (0–0.35 M). Pure PON1 was obtained from the above-mentioned PON1/HPBP preparations using a specific hydroxyapatite chromatography step. The PON1/HPBP fractions issued from ion exchange step were loaded on a glass column (1 cm \times 10 cm, Amersham) containing 2 ml Bio-Gel HTP hydroxyapatite (BioRad Laboratories, Munich, Germany). Hydroxyapatite was equilibrated with 10 mM sodium phosphate pH 7.0 without Triton. After washing, bound proteins were eluted by increasing linearly the ionic strength of the buffer from 10 to 400 mM sodium phosphate (pH 7.0). Equilibration and elution buffers were calcium free. HPBP was not retained on hydroxyapatite, and was collected in the filtrate. On the contrary, PON1 was retained and subsequently eluted by means of phosphate ions. Purified preparations were stored in 25 mM Tris buffer pH 8.0, 1 mM CaCl₂, containing 0.1% Triton. Two molar ratios of PON1 to HPBP (9:1 and 1:1) were selected. The human enzyme preparations in 25 mM Tris–HCl, 1 mM CaCl₂, 0.1% Triton X-100, at pH 8.0 were stored at –20 °C in aliquots of 500 μ L. Protein concentration was determined by the bicinchoninic acid protein assay (BCA kit from Interchim, Montluçon, France), with BSA as a standard. The chimeric mammalian recombinant PON1 (rPON1) dubbed G3C9-8His, obtained using directed evolution *via* gene shuffling of human, rabbit, rat and mouse PON1 genes was a gift from Dr. Dan S. Tawfik (The Weizmann Institute of Science, Rehovot, Israel). This evolved mammalian rPON1 was purified as previously described [4], and stored at 4 °C in 50 mM Tris, 50 mM NaCl, 1 mM CaCl₂, 0.1% Tergitol, at pH 8.0.

2.3. Capillary electrophoresis

CE analyses were performed using a Beckman Coulter P/ACE 5510 system (Beckman Gagny, France) equipped with a diode array detector, and a P/ACE Station 1.21 software for system control, data collection and data analysis. This system was modified for strict temperature control, as previously described [17]. The improvement allowed both efficient control, with temperature fluctuations lower than 0.05 °C, and accurate measurement of the temperature (± 0.1 °C) in close proximity to the outer capillary wall. Bare fused-silica capillaries (50 μ m i.d. \times 67 cm) were used. Sequential electrophoretic migrations at 30 °C, were performed with the usual polarity conditions, at a constant voltage. The capillary was conditioned before each series of analysis with 1N sodium

hydroxide (10 min), followed by distilled water (10 min) and a final 5 min equilibrium with running buffer (borate 250 mM pH 9.0). Prior each sample injection, the capillary was flushed with the same successive solutions for 2, 2 and 5 min, respectively. These washings were intended to provide convenient reproducibility of the EOF and thus, that of the migration time. The protein samples (1 mg/ml) complemented with DMF (0.01% v/v final concentration) as the EOF marker, were injected hydrodynamically by overpressure (3.45 kPa). Acquisition of absorbance data in the range 195–300 nm was performed at 2 Hz using the diode array device. Electropherograms were obtained at a single wavelength (200 nm). The mass detection limit is $\sim 10^{-13}$ – 10^{-16} moles, and the concentration detection limit is $\sim 10^{-5}$ – 10^{-8} M [18]. The mobility of proteins (μ) was calculated through the software from the apparent migration times, as follows:

$$\mu = \mu_{\text{app}} - \mu_{\text{eo}} = \frac{L_t L_d}{V} \left(\frac{1}{t_{\text{app}}} - \frac{1}{t_{\text{eo}}} \right) \quad (1)$$

whereas μ , μ_{app} and μ_{eo} are the electrophoretic, apparent and electroosmotic mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$), respectively. L_t is the total length of the capillary and L_d the length of the capillary from inlet to detection window; V is for the applied voltage, t_{app} the apparent migration time and t_{eo} the migration time of the EOF marker. Surfaces of overlapping peaks were fitted after deconvolution (using PeakFit v4 software, SPSS Science, Chicago, IL) with the Symmetric Double Gaussian Cumulative function.

2.4. Determination of enzymatic activities

The experimental apparatus for kinetic measurements under pressure (P) was made of a hand screw pump (TOP Industrie, Vaulx le Penil, France) and an optical pressure vessel equipped with sapphire windows (SOFOP, Olemps, France). This vessel was interfaced to a Cary I (Varian, Les Ulis, France) spectrophotometer and can operate up to 500 MPa. Arylesterase, lactonase and OPH activities were assayed at 25 °C in 50 mM Tris, 1 mM CaCl_2 , pH 8.0 for hydrolyses of phenyl acetate (PhA), 2-coumaranone (2-Coum) and paraoxon (PO), respectively. Enzymatic parameters (k_{cat} , K_m and/or k_{cat}/K_m) with PhA and PO were determined by Michaelis–Menten analysis of steady-state kinetics. Kinetic data with 2-Coum were fitted to the linear portion of the Michaelis–Menten plot ($[S] \ll K_m$) as well as for hydrolysis of the three substrates by PON1/HPBP (1:1). The final substrate concentrations were 0.1–7 mM for PhA, 0.1–2 mM for 2-Coum and 0.5–7 mM for PO in the presence of 7, 2.5, and 3% isopropanol, respectively.

The hydrolysis kinetics were followed in the pressure range 0.1–250 MPa, by recording the increase in absorbance at 270, 274 and 412 nm, for hydrolysis of PhA, 2-Coum and PO, using 3439, 1715 and $13,486 \text{ M}^{-1} \text{cm}^{-1}$, respectively, as the molar extinction coefficient, ϵ . The ϵ of chromogenic substrates does not significantly vary in the pressure range used. Tris buffer was chosen because its pH is pressure-invariant. For all studied PON1s, no spontaneous hydrolysis of PhA and PO was observed. Rates of spontaneous hydrolysis of 2-Coum at atmospheric pressure (P_{atm}), increasing under pressure, were subtracted from all measurements. Apparent steady-state velocities were recorded after 5 min reaction, a delay dictated by experimental constraints. Pressure-induced changes in catalytic activity were followed by plotting reaction rates as a function of substrate concentrations at each pressure. For kinetics carried out at low $[S]$, i.e., $[S] \ll K_m$, the activation volume associated with binding and kinetic step ($\Delta V_{k_{\text{cat}}/K_m}^\ddagger$), was determined from the pressure dependence of k_{cat}/K_m , [19] according to the equation:

$$\left(\frac{\partial \ln k_{\text{cat}}/K_m}{\partial P} \right)_T = - \frac{\Delta V_{k_{\text{cat}}/K_m}^\ddagger}{RT} \quad (2)$$

whereas R is the gas constant ($82 \text{ ml} \times \text{atm} \times \text{K}^{-1}$) and T , the temperature in Kelvin.

When kinetics were carried out over a wide substrate range up to saturation, the activation volume ($\Delta V_{k_{\text{cat}}}^\ddagger$) and the binding volume change (ΔV_b), were determined ($\Delta V_b = -\Delta V_{K_m}$ because K_m can be regarded as a dissociation constant). Thus, volume changes upon binding and activation volume were determined from the pressure dependence of K_m and k_{cat} , respectively according to the following equations:

$$\left(\frac{\partial \ln K_m}{\partial P} \right)_T = - \frac{\Delta V_{K_m}}{RT} \quad (3)$$

and

$$\left(\frac{\partial \ln k_{\text{cat}}}{\partial P} \right)_T = - \frac{\Delta V_{k_{\text{cat}}}^\ddagger}{RT} \quad (4)$$

3. Results and discussion

3.1. Cofactors for in vitro stabilization of active PON1

In this study are reported some results of our integrative approach, including spatial and temporal parameters to better understand the mechanisms of action of PON1 in a complex medium. Our attempt deals with several cofactors (e.g., ions, protein, and inhibitor). Calcium and phosphate ions were investigated as intrinsic or extrinsic protein cofactors. Indeed, two calcium ions are present in PON1. One is solvent-exposed (the catalytic calcium) and located at the top of the central tunnel of the six-bladed β -propeller structure. The second Ca^{+2} is buried at the center of the tunnel and predicted to maintain the overall structure of the protein. A phosphate ion is found bound to the catalytic calcium in the structure of crystallized rPON1 G2E6. This phosphate coming from the mother liquor of the crystal may mimic the binding of the phosphate group of the phosphotriester substrates. Calcium and phosphate ions can also act as extrinsic cofactors present in blood. The concentration of calcium in plasma is 2.25–2.65 mmol/L, suggesting that PON1 should be fully active *in vivo*. The concentration of phosphate in plasma is 0.8–1.5 mmol/L. The second player is HPBP, considered as a possible transient chaperone. Finally, an inhibitor of PON1, 2-hydroxyquinoline (2-HQ) was investigated as a possible active site stabilizer (as several stabilizing ligands were shown for cholinesterases [20,21]). For investigating the effects of these factors on stability and activity of PON1, we used different approaches, including kinetics under high pressure, and analysis of oligomeric states by CE.

3.2. High pressure for analyzing the protein environment-dependence of PON1 activities

The functional stability of PON1s at P_{atm} was first investigated by kinetic analysis of the catalytic efficiency (k_{cat}/K_m), using PhA, 2-Coum and PO as the substrates (Table 1). The arylesterase k_{cat}/K_m was of the same order ($\sim 10^6 \text{ min}^{-1} \text{M}^{-1}$) for all PON1 preparations. The lactonase and OPH activities of free PON1 were very low and not detectable. All catalytic activities were stabilized by the presence of 10% HPBP. The k_{cat}/K_m value of rPON1 was of the same order for both arylesterase and lactonase activities and lower for OPH activity. The catalytic behavior of rPON1 is similar to that of various PON1/HPBP preparations, but k_{cat}/K_m values were higher. These data confirmed previous statement that the enzyme needs a chaperone (in the case of PON1 [13,24]) or to be mutated to favor solubility/stability (in the case of rPON1 [4]), for catalytic activities. These experiments provided evidence that: (i) the arylesterase activity of PON1 was more resistant to structural instability than

Table 1Catalytic efficiency (k_{cat}/K_m in $\text{min}^{-1} \text{M}^{-1}$) of arylesterase, lactonase, and OP-hydrolase activities, for the different PON1 formulations at atmospheric pressure.

Activity	Substrate	PON1	PON1/HPBP (9:1)	PON1/HPBP (1:1)	rPON1
Arylesterase	Phenyl acetate	$3.2 \pm 0.4 \times 10^6$	$6 \pm 0.3 \times 10^6$	$4 \pm 0.3 \times 10^6$	$8. \pm 0.5 \times 10^6$
Lactonase	2-Coumaranone	ND ^a	$0.8 \pm 0.1 \times 10^6$	$4.4 \pm 0.4 \times 10^6$	$7.9 \pm 0.4 \times 10^6$
OP-hydrolase	Paraoxon	ND ^a	$2 \pm 0.2 \times 10^3$	$1.3 \pm 0.3 \times 10^3$	$92 \pm 7 \times 10^3$

Activity assays were performed in 50 mM Tris/1 mM NaCl, pH 8.0 at 25 °C. k_{cat}/K_m values are mean \pm SD from at least three experiments.^a ND: non-detectable, due to instability of free PON1.

the other two activities; (ii) that HPBP binding allowed restoring lactonase and OPH activities; and (iii) rPON1 is more stable than the other enzyme preparations. The first result brings an additional proof that the active sites responsible for PON1 activities do not overlap [5,6,25]. The second result suggests further investigation to understand the stoichiometry of PON1/HPBP relationships. The third observation suggests reservations about the validity of hybrid mammalian rPON1s to reflect the conformational and functional behaviors of the human enzyme. A recent work showing dramatic differences in OPH activity between human PON1 and chimeric mammalian rPON1 [26], confirmed our opinion that hybrid rPON1s does not mimic perfectly human PON1 catalytic behavior [16].

3.3. Pressure-withstanding of catalytic parameters of PON1s

The pressure dependence of PON1s' catalytic activity was investigated by analyzing the pressure dependence of $\ln(k_{\text{cat}}/K_m)$ for arylesterase, lactonase and OPH activities of different PON1 preparations in the range 1–250 MPa. Plots were biphasic for both arylesterase and lactonase activities, leading to two apparent activation volumes (ΔV^\ddagger), depending on the pressure range (i.e., below and above the pressure break, P_t) (Fig. 1). Biphasic plots showed that for P up to P_t , catalysis of PON1, PON1/HPBP (9:1), and rPON1 was somewhat unaffected ($\Delta V^\ddagger \approx 0$). On the contrary, above P_t , a pressure-induced inhibition of both activities for the three PON1s was observed, and ΔV^\ddagger were positive. Unlike the other preparations, the arylesterase and lactonase activities of PON1/HPBP (1:1) were favored by P up to P_t and slightly disfavored by P above P_t . In this PON1 formulation, lactonase and arylesterase activities were constant up to 150 and 250 MPa, respectively (data not shown). The pressure dependence of $\ln(k_{\text{cat}}/K_m)$ for OPH activity of rPON1 and PON1/HPBP (9:1 and 1:1) differed considerably. The biphasic catalytic behavior of rPON1 showed that reaction was slightly modulated up to 250 MPa (data not shown), the reaction being rather favored by P below P_t (at 100 MPa) and slightly disfavored by P above P_t . On the contrary, the pressure dependence of $\ln(k_{\text{cat}}/K_m)$ for OPH activity of PON1/HPBP preparations

was linear up to 200 MPa (data not shown). The observed negative ΔV^\ddagger indicated that reaction is favored by pressure (≈ 5 -fold more active at 200 MPa than at P_{atm}). Volume changes upon substrate binding ($\Delta V_b = -\Delta V_{k_m}$) and activation volumes $\Delta V_{k_{\text{cat}}}^\ddagger$, associated with hydrolysis of different substrates under pressure were determined. Experimental constraints allowed their determination only for PhA and PO hydrolysis by rPON1 and PON1/HPBP (9:1) and PhA hydrolysis by PON1. The plots $\ln k_{\text{cat}}$ vs. P were linear up to 150 MPa regardless the enzyme substrate. The plot for OPH activity of rPON1 was biphasic, with a negative activation volume up to P_t (at 150 MPa), followed by a slightly positive activation volume (Supplemental Fig. S1). Likewise, the plots $\ln K_m$ vs. P were different for arylesterase and OPH activities. Binding of PhA was insensitive to P up to P_t at 125 MPa for both rPON1 and PON1/HPBP (9:1), and at 175 MPa for PON1, respectively (Supplemental Fig. S2). For this substrate, all plots were biphasic and K_m increased with P above P_t . Negative ΔV_{K_m} indicates that the binding step was disfavored, whatever the PON1 preparation. Under similar conditions, the pressure dependence of K_m for the OP substrate was different for rPON1 and PON1/HPBP (9:1). At $P < P_t$, ΔV_{K_m} (associated with the binding of PO to rPON1) was negative and at $P > P_t$ a relatively invariant K_m was observed. This pressure break suggests that a new active conformation appears beyond P_t (at 150 MPa). The K_m value for hydrolysis of PO by PON1/HPBP (9:1) decreased linearly with P , indicating that affinity for this substrate was favored by P ($\Delta V_b < 0$). Finally, the present work shows that pressure enhances the activity and/or modulates the specificity of PON1, whatever the catalytic mechanism, regardless the type of substrate.

Pressure is known to induce changes in size of protein cavities, alteration in protein hydration [27–29], and changes in catalytic behavior of enzymes [19]. Such pressure-induced effects were expected for PON1. Although incomplete, due to the lack of large amounts and/or instability of enzyme preparations, this study allowed dissecting the destabilization of the three enzyme activities for different PON1 preparations. The activation volumes and volume change below and beyond the pressure breaks (P_t), associated with catalytic efficiency ($\Delta V^\ddagger k_{\text{cat}}/K_m$), catalytic step ($\Delta V_{k_{\text{cat}}}^\ddagger$)

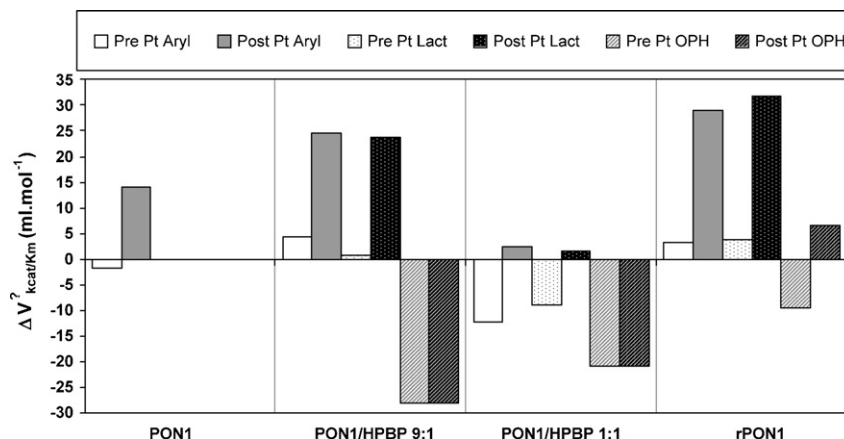


Fig. 1. Pressure dependence of the catalytic efficiency of PON1 preparations: activation volume associated with binding and kinetic step ($\Delta V^\ddagger k_{\text{cat}}/K_m$) for arylesterase, lactonase and OPH activities (from left to right) for PON1, PON1/HPBP (9/1), PON1/HPBP (1/1) and rPON1. The values of activation volume (in $\text{ml} \times \text{mol}$) determined below and above P_t , the pressure breaks, are shown as light and dark symbols, respectively.

and substrate binding step (ΔV_b), for arylesterase, lactonase and OPH activities is the first description of the pressure effects on PON1 catalytic activities. Again, results strengthen the fact that PON1 displays two distinct catalytic behaviors, i.e., an arylesterase and lactonase activities, and an OPH activity. It was postulated a single active site could be responsible for hydrolysis of several substrates, the differences being likely due to the mode of binding [30]. Human PON1 and bacterially expressed chimeric rPON1s (G2E6 which provided crystal structure, and G3C9) differ by multiple amino acids, none of which being in the putative enzyme active site. At the moment, there is no catalytic mechanism described for human PON1. A reaction mechanism was approached for rPON1s [5–7], and using a structural model of human PON1 based on the crystal structure of the rPON1 G2E6 [31]. A mechanism study for the OPH activity of the diisopropyl fluorophosphatase (DFPase) from the squid *Loligo vulgaris* having a six-bladed β -propeller scaffold resembling the rPON1, suggested that D269 may function as a nucleophile in PON1 [32]. Since DFPase has no helices at the top of the propeller forming a close active site, this assumption has to be regarded cautiously. H115 was proposed to be a residue involved in binding of OP substrates [25]. Indeed, H115W mutation altered the capacity of certain OPs to occupy the active site in proper position. Esters and lactones may bind to another subsite than OPs. Recently, *in silico* studies of substrate interactions with PON1, suggested the existence of two distinct binding regions, one site (involving a part of helix H2 and residues R/Q192, Y71, H115 and H134) for arylesters/lactones and the other (involving a part of helix H3, and residues F292, D269) for phosphotriesters [31] (additional information is provided in Supplemental Fig. S3). Our results from high pressure kinetic study, showing that human PON1 displays two distinct catalytic behaviors for OP or esters and lactones, agree with this hypothesis.

3.4. CE for analyzing the oligomeric state-dependence of PON1 stability and activity

3.4.1. Size heterogeneity of PON1

CE is considered as a high resolution analytical technique that can routinely provide 10^5 – 5×10^5 theoretical plates for proteins. The reproducibility of mobility measurements expressed in terms of relative standard deviation (RSD) was less than 0.5%. The reproducibility of migration time is typically 0.5–1%. Accordingly, CE is a pertinent tool for investigating the heterogeneity of proteins. Because of the use of high electric fields in CE and much difference in *pI* between PON1 and HPBP (~5 and ~8, respectively), a clear separation of both proteins in PON1 preparations containing HPBP was possible. As it was previously shown [13], CE showed that PON1 preparations displayed heterogeneous pattern with at least two oligomeric states. In order to facilitate interpretation of the effects of cofactors on PON1 oligomerization, CE conditions were screened to provide parameters allowing optimal analysis of PON1 peaks. For example, high buffer concentration (250 mM borate) and alkaline buffer (pH 9.0) favored sensitivity by reducing wall interactions, capillary dimensions, electric field and temperature control prevent excessive joule effect and provided valuable determination of peak mobility and peak area. This optimization provided electropherograms allowing deconvolution of PON1 peaks issued from CE runs after different incubation times. Then, we used the Offord model (Eq. (5)) that correlates electrophoretic mobility with the charge-to-size parameter [22]:

$$\mu_{ep} = Aq/M^{2/3} \quad (5)$$

whereas μ is the electrophoretic mobility, q the charge and M the molar mass.

An example of this analysis is shown in Fig. 2; a heterogeneous PON1 fraction is regularly seen in capillary electropherograms

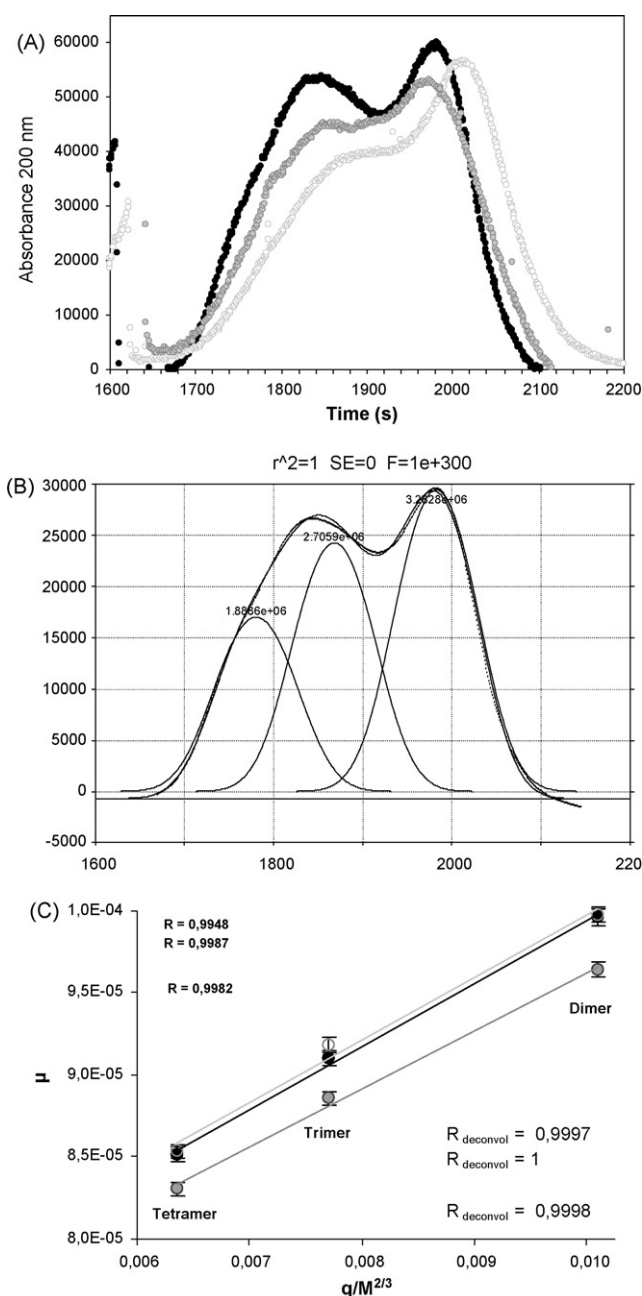


Fig. 2. CE electropherograms to decipher time-scale oligomerization changes of PON1 (A) CE pattern of PON1/HPBP (1:1) (grey, middle curve) and of PON1/HPBP (9:1) after 0 min (dark, upper curve) and 15 days (light grey, lower curve) of incubation with 100 μ M phosphate. Migration was carried out at 30 $^{\circ}$ C in a 50 μ m i.d. \times 67 cm fused-silica capillary, constant voltage 16 kV, and 200 nm detection, in a 250 mM borate buffer pH 9.0. Analysis of PON1 oligomeric state (B) deconvolution of the PON1 peak obtained for CE migration of PON1/HPBP (9:1) after 0 min of incubation with 100 μ M phosphate showed three distinct molecular populations ($R^2_{deconvol} = 1$) whose relative areas were 24.0, 34.3 and 41.7%, respectively (from left to right). Determination of oligomer size (C) plots of electrophoretic mobility μ to the ratio $q/M^{2/3}$ for the three PON1 samples described in (A) allowed characterizing the three molecular populations as being tetramers, trimers and dimers (from left to right).

(Fig. 2A). Peak deconvolution showed that this fraction contained three oligomeric forms (Fig. 2B). Using the Offord relation (Fig. 2C), we showed that these oligomeric forms correspond to dimers, trimers and tetramers, in decreasing order of apparent mobility (and subsequently in increasing order of electrophoretic mobility).

No clear characterization of the oligomeric state of human PON1 has been reported so far. CE was used for separation of PON1's

native state(s) either in the presence or absence of stabilizing compounds. By itself, CE is not meant to provide an estimation of protein molecular size. However, deconvolution of PON1 peaks showed that in all PON1 preparations, three oligomeric forms were present. The percentage of these forms varied with the molecular environment and with time. Besides, it was possible to assign dimeric, trimeric and tetrameric states to the three Gaussian peaks populated after deconvolution. Polydispersity in apparent M_r of PON1 may have several origins. Detergent and buffer composition were shown to affect the oligomeric state of PON1 [8]. Different biophysical techniques confirmed PON1 heterogeneity in preparations containing non-micellar concentrations of detergents, but reported M_r estimations show discrepancies. The experimental impediments and difficulties in data analysis of HDL-associated proteins, together with the strong association of PON1 with HPBP may have contributed to such discrepancies. Size-exclusion chromatography analysis suggested monomer–dimer equilibrium in the presence of 0.6 mM C_{12} -maltoside for rPON1 [4], whereas it showed dimer and tetramer, but provided no evidence for trimer (Elias et al., unpublished results). Ferguson plot analysis, with activity-staining, showed no active monomer. This indicates that the dimer is the first state of oligomerization for active PON1 [16]. A similar analysis for human rPON2 in the presence of 0.05% n-dodecyl- β -D-maltoside showed an active trimer [33]. Our approach, using optimized CE conditions, showed three PON1 oligomeric forms differing in size; one of them being likely a trimer.

3.4.2. Modulation of oligomerization and activity by calcium and phosphate

The analytical strategy described above was incremented for studying the effects of ionic cofactors on the oligomeric state and activity of PON1 as a function of time. The changes in oligomeric states of a PON1 formulation in the presence of 100, 250, and 500 μ M PO_4^{3-} (sodium phosphate) were monitored by performing CE runs after different incubation times. At the same time, enzyme activity was assayed in corresponding samples. Fig. 3, showing electropherograms for different incubation times of PON1 samples containing phosphate at different concentrations illustrates the modulating effect of phosphate ion on oligomerization of the enzyme. 100 μ M phosphate (Fig. 3A) first modified the initial oligomeric equilibrium and progressively favored dissociation of heavy forms into dimers. Similar patterns were observed with 250 and 500 μ M phosphate (Fig. 3B and C). The overall effect of phosphate ion was a propensity to slowly promote dissociation of heavy oligomeric forms of PON1. A similar study was conducted with a PON1 preparation in the presence of 500, 750 and 900 μ M Ca^{2+} (Fig. 4). While the two former concentrations provided patterns

similar to those observed with phosphate (Fig. 4A and B), i.e., a regular dissociation of heavy oligomers, the later failed to cause a clear change in the oligomeric distribution (Fig. 4C). Finally, both ionic effectors were shown to modulate the oligomerization of PON1 in the same way.

In order to estimate the possible effect of phosphate and calcium ions, on the catalytic activity of the enzyme, a preliminary approach was opened by following the time-course of arylesterase, lactonase and OPH activities of PON1 in the presence of 100 μ M phosphate (Fig. 5). Both lactonase and OPH activities are maintained by phosphate. Arylesterase activity is also preserved with considerable fluctuations. All activities were preserved after 1-month storage at 4 °C.

Phosphate and calcium concentrations were chosen to provide enzyme/ionic cofactor ratios below, within and above their physiological plasma concentrations, according to PON1 concentration in our assays. This provided evidence that PON1 oligomerization was affected by phosphate and calcium ions and that the observed changes in oligomers distribution varied with time, with a common propensity to dissociation of heavy forms into dimers, except at the highest concentration (900 μ M) of calcium. Ca^{2+} , the natural structural and catalytic cofactor of PON1 active site appears to favor the presumed naturally occurring PON1 oligomer state, i.e., the dimer. Phosphate is an extrinsic PON1 cofactor, but may bind to the catalytic Ca^{2+} as a mimic of the phosphyl-moiety of OP substrates. Binding of phosphate shifted the oligomer equilibrium towards dimers. This possible stabilizing effect on “natural”-like PON1 oligomer state fits with the frequent conformational stabilization of enzymes active site by ligands. At the same time, we followed the time-course of arylesterase, lactonase and OPH activities of PON1, in the presence of 100 μ M phosphate. After initial fluctuations in the first time period (0–300 min), corresponding to that analyzed by CE, all activities were maintained for a month, with persistent fluctuations of arylesterase activity. This suggests that phosphate ion affected differently arylesterase and lactonase/OPH activities. This result strengthens the hypothesis of non-overlapping active sites of PON1 (see Section 3.3).

3.4.3. Modulation of oligomerization and activity by HPBP in the presence of phosphate

The changes in oligomeric states and of enzyme activity of a PON1 preparation in the presence of HPBP at two molar ratios, i.e., 9:1 and 1:1, were monitored by performing CE migrations after different incubation times (Fig. 6A), and by measuring arylesterase activity of corresponding samples (Fig. 6B). While PON1/HPBP (9:1) was unable to prevent phosphate-induced dissociation of heavy PON1 oligomers, the equimolecular 1:1 ratio provided stabiliza-

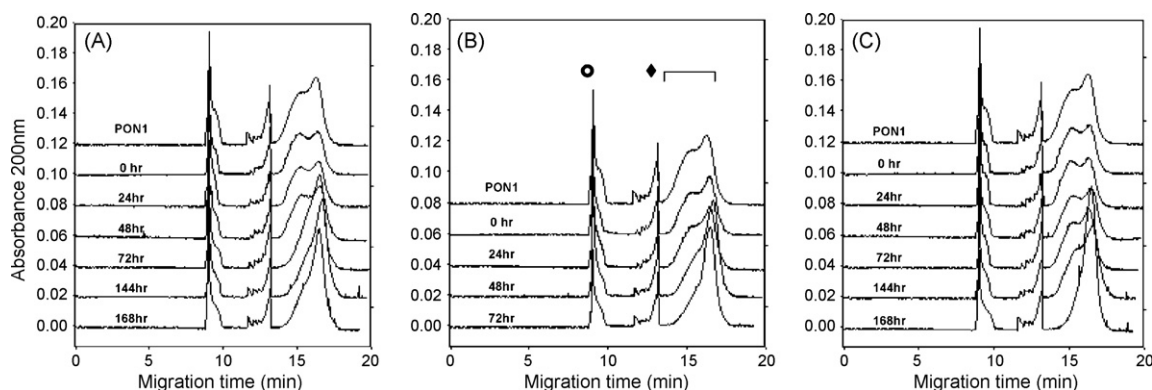


Fig. 3. Electropherograms for the time-course of PON1 supplemented with phosphate: (A) 100 μ M, (B) 250 μ M and (C) 500 μ M of sodium phosphate were added to PON1, and submitted to CE migrations after different reaction times. The 1st (●), 2nd (◆) and 3rd (□) (broad) peaks correspond to DMF (the EOF marker), Tris (present in the sample buffer) and PON1, respectively.

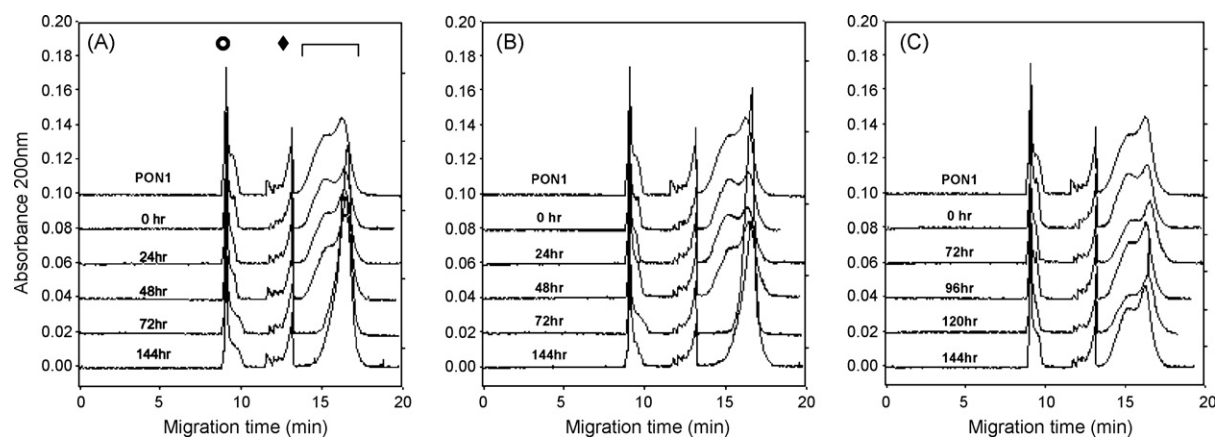


Fig. 4. Electropherograms for the time-course of PON1 supplemented with calcium: (A) 500 μ M, (B) 750 μ M and (C) 900 μ M calcium chloride were added to PON1, and submitted to CE after different reaction times. The 1st (●), 2nd (◆) and 3rd (□) (broad) peak correspond to DMF (the EOF marker), Tris (present in the sample buffer) and PON1, respectively.

tion of the initial oligomer equilibrium. Measurements of residual arylesterase activity in corresponding aliquots showed that the equimolecular ratio also prevented the early negative effect of phosphate on activity. Finally, HPBP is needed for structural and functional stabilization of PON1 preparations.

The scrutiny of PON1 oligomerization by exploitation of CE data, with concomitant exploration of catalytic activity was applied to estimate the influence of the PON1/HPBP ratio on the modulating effect of 100 μ M phosphate. Compared to the oligomers pattern in the absence of phosphate, the PON1/HPBP (9:1) preparation showed no significant change with time for the dimer proportion ($\approx 42\%$, as estimated by deconvolution), but a tendency to disfavor tetramer proportion. Examination of the oligomers pattern of PON1/HPBP (1:1) showed a clear increase and stabilization of dimer proportion at $\approx 50\%$, at the expense of trimers (data not shown). Stabilization of PON1 by equimolecular concentration of HPBP under dimeric form was also illustrated by the arylesterase activity that is maintained at a level close to the maximum. This confirmed that stabilization of PON1 requires HPBP as a chaperone, and that the preferred oligomeric state of the enzyme is the dimer. Because CE separates PON1 from HPBP due to their much different *pI*, the functional dimers characterized in the present study are homodimers.

3.4.4. Modulation by phosphate and calcium ions of the inhibitory effect of 2-HQ

Because calcium and phosphate are deeply associated to PON1 active site, and since 2-HQ was shown to interact at the entrance of the enzyme active site [23], an additional approach for understanding the mechanism(s) of oligomerization/activity modulation by the phosphate and calcium cofactors was developed, by using

the reversible active site inhibitor 2-HQ. The time-course of the ionic cofactors (at one concentration) effect on PON1 inhibition by 2-HQ was investigated, by estimating oligomer distribution, and measuring the arylesterase activity. The study was performed using PON1/HPBP (1:1) preparation. Whereas direct visual examination of electropherograms suggested minute changes in oligomerization, peak deconvolution provided evidence for distinct effects. 2-HQ had no clear effect on oligomer distribution in the presence of phosphate (Fig. 7A). On the contrary, calcium was shown to favor formation of PON1 dimers (Fig. 7B). Inhibition of PON1 arylesterase activity was also differently affected by phosphate⁻ and calcium (Fig. 7C). Phosphate had no immediate effect, allowing $\sim 70\%$ inhibition of the enzyme activity. Then, full activity was gradually recovered as a function of time. In contrast, calcium allowed quasi instantaneous recovery of total enzyme activity.

To complete the scrutiny of the effects of cofactors on activity and stability of PON1, we explored the role of 2-HQ. The rationale is that: (i) 2-HQ is a selective competitive inhibitor of PON1's activities [3,30,34] and (ii) HQs are lactams, i.e., isosteric forms of lactones in which nitrogen replaces the ring oxygen. Thus, HQs are not hydrolyzed by PON1 but inhibit it. On the other hand, reversible active site inhibitors were described as being able to stabilize conformation of enzymes [20,21]. The observed inhibition by 2-HQ of arylesterase activity of purified PON1 ($>70\%$), was similar to that determined in HDL (60 and 70% for arylesterase and OPH activity, respectively [35,36]) or in crude serum (83–95% [34]). The effect of calcium and phosphate ions on inhibition by 2-HQ carried out on PON1/HPBP (1:1) formulation supposedly able to provide maximal stabilization of the enzyme. Calcium or phosphate ions failed to induce visible changes in oligomer distribution of PON1 inhibited

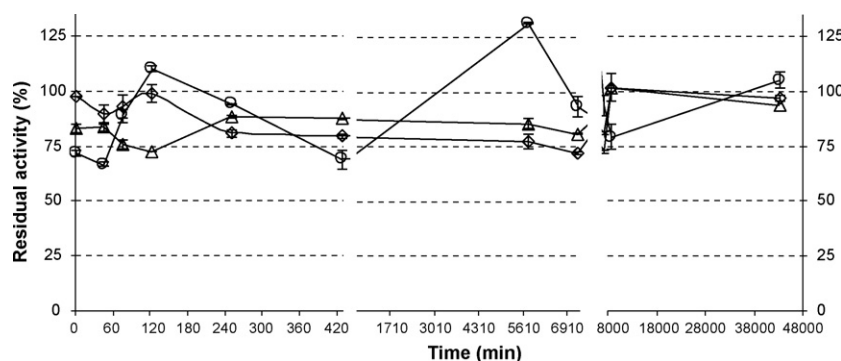


Fig. 5. Stability of arylesterase (●), lactonase (◇) and OPH (▽) activities of PON1 in the presence of 100 μ M phosphate, during storage at 4 °C.

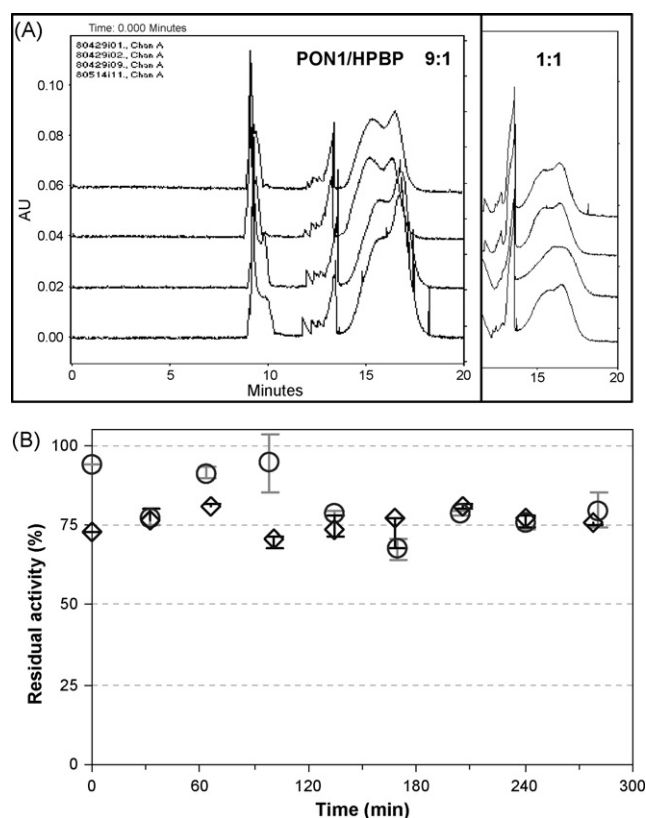


Fig. 6. Effect of HPBP on time-course oligomerization and activities of PON1 in the presence of phosphate 100 μ M: (A) CE pattern after different reaction times with phosphate, of PON1/HPBP (9:1) and (1:1). (B) Corresponding residual arylesterase activity of PON1/HPBP (9:1) (diamonds) and (1:1) (circles).

by 2-HQ, under our experimental conditions. However, the enzyme displayed distinct catalytic behaviors. Calcium allowed immediate recovery of full activity, unlike phosphate that delayed recovery of full activity for ~ 2 h. “Inhibition of the inhibitor” by calcium is likely due to competition between extrinsic calcium ion and 2-HQ for the enzyme active site. Another HQ (8-HQ) was shown to chelate transition metal cations (Zn^{2+} , Co^{2+} , Mn^{2+} , Cd^{2+} , Ni^{2+}) in bacterial OPH [37], causing only partial inhibition of the enzyme [38]. However, chelating effect of 8-HQ on alkaline earth metals (e.g., Mg^{2+} , Ca^{2+}) is debated [39–41]. Finally, there is no clear evidence for calcium chelating effect of 2-HQ.

The concept that emerged from our study can be summarized as follows: addition of phosphate ion provokes a transient decrease in activities. This could be due to competition of the added phosphate with the substrate (a phosphate ion was found at 2.2 Å from the catalytic calcium in the crystallized rPON1 [5]). A possible chelating effect of 2-HQ is suggested by the fact that addition of calcium favored the supposed “natural” active dimers and quickly restored activity of the enzyme inhibited by 2-HQ. A recent investigation of mutated positions in 2-HQ-resistant rPON1 variants showed that most of the mutated residues form a continuous patch [23]. Three of these residues are part of the active site wall opposite to the H115–H134 dyad area; the other lay beneath the active site, one being solvent-exposed, adjacent to the active site cleft and close to the N-terminal helix (Fig. 8). This configuration argues for a possible interaction of the inhibitor with residues involved in access to the active site and catalytic activity. These residues form a canopy that controls access of substrates and inhibitors.

In addition to the snapshots of enzyme structures frozen in crystal, molecular dynamics study of PON1 in action is mandatory. Our

approach is a step to evaluate the contribution of cofactors to the functional activity of PON1. On the one hand, calcium was characterized by the mechanism and stability of the enzyme that was considered as a monomer. On the other hand, the minimal modular architecture of functional PON1 is a homodimer. It is of interest to re-examine PON1–calcium interactions in the true physiological oligomeric state of the enzyme. It is worth noting that in the structure of some six-bladed β -propellers, one or two additional calcium ions are located on the surface, and involved in stabilization of homodimers [42,43]. To date, crystallization of human PON1 has failed. Attempts at understanding its catalytic mechanisms are mostly supported by data provided by hybrid rPON1s. However, these enzymes do not mimic the true functioning of the human enzyme [26]. This argues for developing synergistic approaches capable to provide exhaustive information for char-

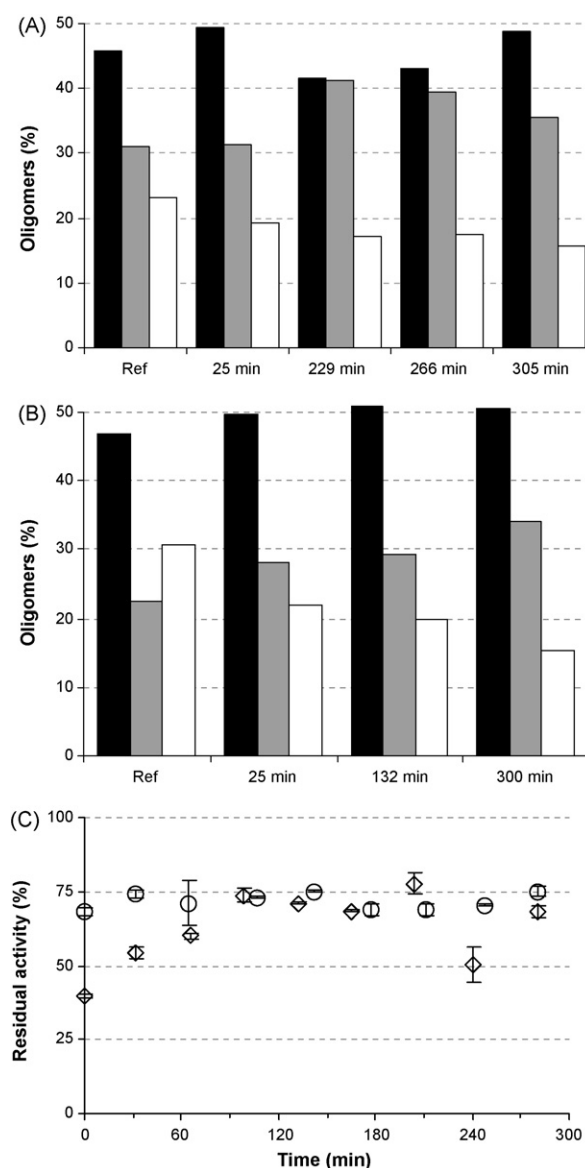


Fig. 7. Effect of phosphate and calcium on PON1 inhibition by 2-HQ: (A) oligomer distribution after deconvolution of PON1 peak into dimers (black), trimers (grey) and tetramers (white) (from left to right). Concentrations were 40 μ M PON1, 100 μ M phosphate, 50 μ M 2-HQ. (B) Oligomer distribution after deconvolution of PON1 peak into dimers (black), trimers (grey) and tetramers (white) (from left to right). Concentrations were 40 μ M PON1, 750 μ M calcium, 50 μ M 2-HQ. (C) Corresponding residual arylesterase activity of PON1 inhibited by 2-HQ in the presence of 100 μ M phosphate (diamonds) and 750 μ M calcium (circles).

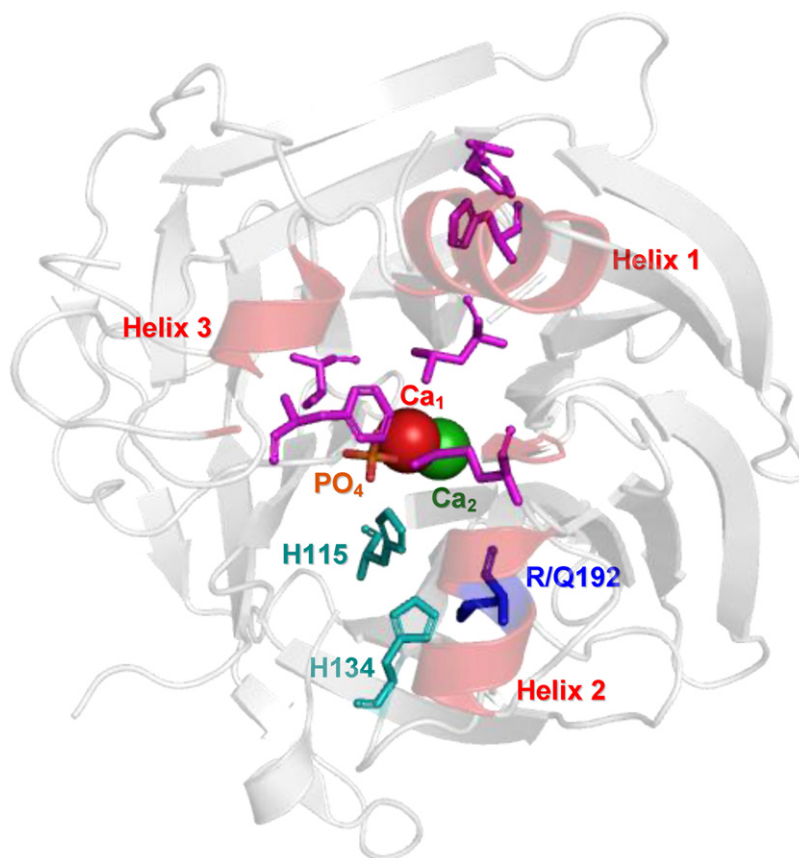


Fig. 8. Positions of PON1 residues involved in interaction with 2-HQ: overall top view of the 3D structure of a rPON1 showing the 3 helices forming the canopy that controls access of substrates to the active site, Ca₂ the structural Ca²⁺ (green sphere), Ca₁ the catalytic Ca²⁺ (red sphere) topped by the phosphate ion (sticks), the polymorphic R/Q192 site (dark blue sticks) and the His115–His134 dyad (cyan sticks). Others residues shown as (magenta) sticks are those whose mutation provided resistance to inhibition by 2-HQ [23]. (For interpretation of the references to color in the citation of this figure, the reader is referred to the web version of the article.)

acterizing parameters involved in the physiological behavior of human PON1.

4. Conclusion

PON1 is recognized to display multiple functions in a complex milieu. The statements that HPBP has a firm tendency to associate with PON1 and is involved in maintaining physiologically active PON1 conformation(s) have steered novel directions for characterizing PON1 functional state(s). As a result, deciphering the requirements for *in vivo* effective PON1 as catalytic bioscavenger is mandatory. This will provide new approaches for generating therapeutic human PON1 intended for prophylaxis or pre-treatments, emergency and post-exposure treatments of intoxications by OP nerve agents. For this arduous task, we used an integrative analytical approach including spatial and temporal parameters. Results showed that *in vitro*: (1) PON1 displays two distinct catalytic behaviors, i.e., arylesterase and lactonase activities, and OPH activity; (2) PON1 functional states are modulated by the molecular environment; (3) PON1 enzyme activities (arylesterase, lactonase, OPH) are differently modulated by this environment; (4) PON1 exists in several active multimeric forms; (5) the binding of HPBP amends the size of the oligomeric states and exerts a stabilizing effect on the activities of PON1; (6) PON1 functional properties (such as oligomerization and activity) are modulated by HPBP, calcium and phosphate. Relevance of these data to understand *in vivo* physiological PON1 functioning will be a next essential goal.

Acknowledgements

The authors thank Dr. Dan S. Tawfik (The Weizmann Institute of Science, Rehovot, Israel) for providing the mammalian rPON1-G3C9. This work was supported by DGA contract (PEA 010807) to P.M. D.R. is under contract with the German Bundesministerium der Verteidigung (M/SABX/8A001).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.11.027.

References

- [1] B.N. La Du, Nat. Med. 2 (1996) 1186.
- [2] D. Rochu, E. Chabrière, P. Masson, Toxicology 233 (2007) 47.
- [3] S. Billecke, D. Draganov, R. Counsell, P. Stetson, C. Watson, C. Hsu, B.N. La Du, Drug Metab. Dispos. 28 (2000) 1335.
- [4] A. Aharoni, L. Gaidukov, S. Yagur, L. Toker, I. Silman, D.S. Tawfik, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 482.
- [5] M. Harel, A. Aharoni, Gaidukov, B. Brumshtein, O. Khersonsky, R. Meged, H. Dvir, R. Ravelli, A. McCarthy, L. Toker, I. Silman, J. Susman, D.S. Tawfik, Nat. Struct. Mol. Biol. 11 (2004) 412.
- [6] O. Khersonsky, D.S. Tawfik, J. Biol. Chem. 281 (2006) 7649.
- [7] M. Rosenblatt, L. Gaidukov, O. Khersonsky, J. Vaya, R. Oren, D.S. Tawfik, M. Aviram, J. Biol. Chem. 281 (2006) 7657.
- [8] D. Josse, C. Ebel, D. Stroebel, A. Fontaine, F. Borges, A. Echalié, D. Baud, F. Renault, M. Le Maire, E. Chabrières, P. Masson, J. Biol. Chem. 277 (2002) 33386.
- [9] A. Fokine, R. Morales, C. Contreras-Martel, P. Carpentier, F. Renault, D. Rochu, E. Chabrière, Acta Cryst. D59 (2003) 2083.

- [10] R. Morales, A. Berna, P. Carpentier, C. Contreras-Martel, F. Renault, M. Nicodeme, M.L. Chesne-Seck, F. Bernier, J. Dupuy, C. Schaeffer, H. Diemer, A. Van-Dorselaer, J.C. Fontecilla-Camps, P. Masson, D. Rochu, E. Chabriere, *Structure* 14 (2006) 601.
- [11] H. Diemer, M. Elias, F. Renault, D. Rochu, C. Contreras-Martel, C. Schaeffer, A. Van Dorselaer, E. Chabrière, *Proteins* 71 (2008) 1708.
- [12] F. Renault, E. Chabrière, J.P. Andrieu, B. Dublet, P. Masson, D. Rochu, *J. Chromatogr. B* 836 (2006) 15.
- [13] D. Rochu, F. Renault, C. Cléry-Barraud, E. Chabrière, P. Masson, *Biochim. Biophys. Acta* 1774 (2007) 874.
- [14] T. Vaisar, S. Pennathur, P.S. Green, S.A. Gharib, A.N. Hoofnagle, M.C. Cheung, J. Byun, S. Vuletic, S. Kassim, P. Singh, H. Chea, R.H. Knopp, J. Brunzell, R. Geary, A. Chait, X.Q. Zhao, K. Elkon, S. Marcovina, P. Ridker, J.F. Oram, J.W. Heinecke, *J. Clin. Invest.* 117 (2007) 746.
- [15] D. Rochu, E. Chabrière, F. Renault, M. Elias, C. Cléry-Barraud, P. Masson, in: M. Mackness, B. Mackness, M. Aviram, G. Paragh (Eds.), *The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism*, Springer, Dordrecht, The Netherlands, 2008, p. 171.
- [16] C. Cléry-Barraud, F. Renault, J. Leva, N. El Bakdouri, P. Masson, D. Rochu, *Biochim. Biophys. Acta* 1794 (2009) 680.
- [17] D. Rochu, G. Ducret, P. Masson, *J. Chromatogr. A* 838 (1999) 157.
- [18] J.P. Landers (Ed.), *Handbook of Capillary Electrophoresis*, CRC Press, New York, 1994, 997.
- [19] P. Masson, C. Balny, *Biochim. Biophys. Acta* 1724 (2005) 440.
- [20] C.S. Payne, M. Saeed, A.D. Wolfe, *Biochim. Biophys. Acta* 999 (1989) 46.
- [21] D. Rochu, C. Cléry-Barraud, F. Renault, A. Chevalier, C. Bon, P. Masson, *Electrophoresis* 27 (2006) 442.
- [22] R.E. Offord, *Nature* 211 (1966) 591.
- [23] G. Amitai, R.D. Gupta, D.S. Tawfik, *HFSP J.* 1 (2007) 67.
- [24] D. Rochu, E. Chabrière, F. Renault, M. Elias, C. Cléry-Barraud, P. Masson, *Biochem. Soc. Trans.* 35 (2007) 1616.
- [25] D.T. Yeung, D.E. Lenz, D.M. Cerasoli, *FEBS J.* 272 (2005) 2225.
- [26] T.C. Otto, C.K. Harsch, D.T. Yeung, T.J. Magliery, D.M. Cerasoli, D.E. Lenz, *Biochemistry* 48 (2009) 10416.
- [27] C. Royer, *Biochim. Biophys. Acta* 1595 (2002) 201.
- [28] B.B. Boonyaratankornkit, C.B. Park, D.S. Clark, *Biochim. Biophys. Acta* 1595 (2002) 235.
- [29] C. Balny, *J. Phys. Condens. Matter* 16 (2004) 1245.
- [30] O. Khersonsky, D.S. Tawfik, *Biochemistry* 44 (2005) 6371.
- [31] X. Hu, X. Jiang, D.E. Lenz, D.M. Cerasoli, A. Wallqvist, *Proteins* 75 (2009) 486.
- [32] M.M. Blum, M. Mustyakimov, H. Rüterjans, K. Kehe, B.P. Schoenborn, P. Langan, J.C.H. Chen, *Proc. Natl. Acad. Sci. U.S.A.* 106 (2009) 713.
- [33] D.I. Draganov, J. Teiber, A. Speelman, Y. Osawa, R. Sunahara, B.N. La Du, *J. Lipid Res.* 16 (2005) 1239.
- [34] O. Khersonsky, D.S. Tawfik, *Chembiochem* 7 (2006) 49.
- [35] M. Rosenblat, R. Karry, M. Aviram, *Atherosclerosis* 187 (2006) 74.
- [36] M. Rosenblat, J. Vaya, D. Shih, M. Aviram, *Atherosclerosis* 179 (2005) 69.
- [37] G.A. Omburo, J.M. Kuo, L.S. Mullins, F.M. Raushel, *J. Biol. Chem.* 287 (1992) 13278.
- [38] F.C.G. Hoskins, J.E. Walker, W.D. Dettbarn, J.R. Wild, *Biochem. Pharmacol.* 49 (1995) 711.
- [39] A. Albert, D. Magrath, *Biochem. J.* 41 (1947) 534.
- [40] L. Prodi, F. Bolletta, M. Montaldi, N. Zaccheroni, P.B. Savage, J.S. Bradshaw, R.E. Izatt, *Tetrahedron Lett.* 39 (1998) 5451.
- [41] G. Farrugia, S. Iotti, L. Prodi, M. Montalti, N. Zaccheroni, P.B. Savage, V. Trapani, P. Sale, F.I. Wolf, *J. Am. Chem. Soc.* 128 (2006) 344.
- [42] C.N. Chen, K.H. Chin, A.H.J. Wang, S.H. Chou, *J. Mol. Biol.* 384 (2008) 604.
- [43] A. Oubrie, H.J. Rozeboom, K.H. Kalk, J.A. Duine, B.W. Dijkstra, *J. Mol. Biol.* 289 (1999) 319.