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Tandem purification of two HDL-associated partner proteins in human plasma, paraoxonase (PON1) and phosphate binding protein (HPBP) using hydroxyapatite chromatography

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Abstract

Human plasma paraoxonase (PON1) is calcium-dependent enzyme that hydrolyses esters, including organophosphates and lactones, and exhibits anti-atherogenic properties. Human phosphate binding protein (HPBP) was discovered as contaminant during crystallization trials of PON1. This observation and uncertainties for the real activities of PON1 led us to re-evaluate the purity of PON1 preparations. We developed a hydroxyapatite chromatography for the separation of both HDL-associated proteins. We confirmed that: (1) HPBP is strongly associated to PON1 in HDL, and generally both proteins are co-purified; (2) standard purification protocols of PON1 lead to impure enzyme; (3) hydroxyapatite chromatography allows the simultaneous purification of PON1 and HPBP.

Keywords: Apolipoproteins; Human phosphate binding protein; Hydroxyapatite; Organophosphates; Paraoxonase

1. Introduction

Human plasma paraoxonase 1 (PON1; aryldialkylphosphatase; EC 3.1.8.1) is a 43–45 kDa calcium-dependent HDL-associated enzyme [1–3]. This enzyme is known for its detoxification and antiatherogenic properties. PON1 hydrolyses numerous organophosphates, including nerve agents (e.g., sarin, soman) and pesticides (e.g., chlorpyrifos oxon, diazoxon), which represent both a terrorist threat and an environmental hazard. Human plasma paraoxonase 1 was also shown to hydrolyse aromatic carboxylic acid esters and lactones [4–6]. Albeit its physiological activity is suggested to be lactonase [6], PON1 is currently considered like an 'enzyme of many substrates' [7] with promiscuous functions [8]. For that reason, some of the activities attributed to PON1 are for the moment largely debated. The three-dimensional (3D) structure and the catalytic mechanism of human PON1 are still unclear. Fine structural and functional studies require purity, but for such an enzyme, purity is a challenge. The particular HDL environment of multiple interacting lipids and proteins may explain why study of paraoxonase in solution is so hard. Besides, all purification procedures of serum PON1 derive from those described at the beginning of the 90s [9,10], which were generally assumed to provide PON1 pure at ≥95%.

In order to solve the 3D structure of human PON1, we used apparently pure and homogeneous enzyme preparations purified following the Gan's protocol [9]. Crystals were obtained from such preparations, but unfortunately the solved structure did not match with the amino-acid sequence of PON1 [11,12].

Abbreviations: ApoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; HPBP, human phosphate binding protein; LCAT, lecithin cholesterol acetyl transferase; OP, organophosphate; PAF-AH, platelet-activating factor acetylhydrolase; PON1, paraoxonase 1

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The solved structure corresponded to an unknown protein, subsequently characterized as a HDL-associated apolipoprotein. This serendipitously discovered protein is a human phosphate binding protein (HPBP) having a MW similar to that of PON1. Co-purified with PON1, HPBP appeared to be a stabilizing partner [13]. In addition, the presence of a phosphate ion bound to the catalytic calcium in the crystal structure of a hybrid recombinant mammalian, PON1 expressed in *E. coli* was unexpected [14]. These observations argue for a possible functional link between PON1 and HPBP, implying phosphate transfer. The 'contaminant' HPBP in allegedly 'pure' PON1 preparation was not regarded as questionable since numerous works reported later the presence of various contaminants in usual PON1 preparations. These contaminants were found to be responsible of certain catalytic activities previously attributed to PON1 [15]. In addition, a recent review highlighted the importance of the HDL-macromolecular environment for the stability and activity of PON1 [16].

Together with the HDL status, the PON1 status is essential in determining susceptibility or protection against toxic compounds or diseases. It also affects the reproducibility of the purification protocols of PON1. Finally, because of the association between PON1 and HPBP, we consistently modified the purification protocol to obtain separately, pure PON1 and pure HPBP.

2. Experimental

2.1. Reagents and materials

Protein standards (HMW and LMW Marker Kits, 14.4–97 kDa) were purchased from Amersham Biosciences (Uppsala, Sweden). Hydroxyapatite media was obtained from Bio-Rad Laboratories (Munich, Germany) as Bio-Gel HTP Hydroxyapatite powder. All other chemicals were of analytical grade, from Sigma–Aldrich Chimie (L'Isle-d'Abeau, France). Centricon 30 concentrators were obtained from Amicon (Danvers, MA).

2.2. Enzyme activity and protein controls

Arylesterase activity of PON1 was determined at 25 °C with phenylacetate (1 mM) as the substrate in 50 mM Tris–HCl buffer pH 8.0, containing 1 mM $CaCl_2$. The rate of hydrolysis was measured spectrophotometrically at 270 nm. The molar extinction coefficient of phenol ($\varepsilon = 1310\ M^{-1}\ cm^{-1}$) was used for calculation of activity. One unit of arylesterase activity is equal to 1 μ mol of phenylacetate hydrolysed/min. Paraoxonase activity of PON1 was determined at 25 °C with paraoxon (1 mM) in 50 mM glycine/NaOH, pH 10.5 buffer containing 1 mM $CaCl_2$. Enzyme assay was based on the estimation at 412 nm of *p*-nitrophenol. The molar extinction coefficient of *p*-nitrophenol ($\varepsilon = 18{,}290\ M^{-1}\ cm^{-1}$ at pH 10.5) was used for calculation of activity. Units of paraoxonase activity are micromoles of paraoxon hydrolysed per minute. Assays were performed using a Helios α spectrophotometer (Unicam Instruments, Cambridge, UK). The protein concentration was estimated using the bicinchoninic acid protein assay Uptima (Interchim, Montluçon, France) with bovine serum albumin as a standard.

2.3. Sample preparation

HPBP/PON1 preparations were obtained as previously described [17] according to a protocol based on the method of Gan et al. [9]. The protocol operated under the same buffer conditions but the ion-exchange matrix was DEAE- or Q-sepharose instead of DEAE bio gel. This was assumed to provide highly purified PON1. Briefly, outdated plasma from blood donors (Établissement Français du Sang Rhône-Alpes, Beynost, France) were supplemented with $CaCl_2$ to a final concentration of 10 mM. This induced clotting at 25 °C; the fibrin clot was separated by filtration. 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 $CaCl_2$ and 3 M NaCl to avoid adsorption of albumin on Cibacron Blue. Elution of hydrophobic proteins, mainly lipoproteins, 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, mainly apolipoprotein A-I (ApoA-I), by anion exchange 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).

2.4. Chromatography on hydroxyapatite

The chromatography system for separations on hydroxyapatite was an ÄKTA FPLC system (Amersham Biosciences) equipped with a computer running UNICORN software (version 3.2) to control the separation unit. Chromatographic runs were performed at 25 °C. The sample was applied in a sample loop. Gradients were mixed using two separate buffers connected to A and B pump modules of P-920. There was an analytical UV flow cell (280 nm) and a conductivity flow cell on line. The active protein fractions issued from ion exchange step were loaded at a flow rate of 0.5 ml min^{-1} on a glass column (1 cm × 10 cm, Amersham) containing 2 ml Bio-Gel HTP hydroxyapatite resin. Hydroxyapatite was equilibrated with 10 mM sodium phosphate pH 7.0 with or without 0.1% Triton (buffer A). After washing with buffer A, bound proteins were eluted by linearly increasing the ionic strength of the buffer from 10 to 400 mM sodium phosphate (pH 7.0), or by direct step at 400 mM phosphate. Equilibration and elution buffers were calcium free. All buffers prepared with milliQ H_2O (0.2 μm) were filtered and degassed before use.

2.5. Electrophoresis

Protein fractions collected during the different purification steps were analyzed by SDS-PAGE performed according to the discontinuous system of Laemmli [18]. The polyacrylamide concentration was 10% (w/v) for the separating gel and 4% for the stacking gel. Prior to loading, samples were incubated in

sample buffer containing 2% (w/v) SDS and 10% (w/v) glycerol, heated for 5 min at 90 °C. Runs were carried out at a constant voltage (200 V) for 45 min. After electrophoresis, proteins were silver-stained using the Bio-Rad Silver Stain Plus kit.

Theoretical titration curves of PON1 and HPBP were built using ABIM, a software resident on the ExPaSy server (http://www.expasy.ch).

2.6. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry analysis

Laser desorption/ionisation mass spectrometric analysis of fractions from hydroxyapatite column was performed with a Perseptive Biosystems (Framingham, MA) Voyager Elite XL time of flight mass spectrometer with delayed extraction, operating with a pulsed nitrogen laser at 337 nm. Positive-ion mass spectra were acquired using a linear, delayed extraction mode with an accelerating potential of 25 kV, a 93% grid potential, a 0.3% guide wire voltage. Each spectrum represents the results from 100 averaged laser pulses. Samples were mixed with an equal volume of a saturated solution of sinapinic acid (Fluka) prepared in a 50% (v/v) solution of acetonitrile/aqueous 0.3% trifluoroacetic acid (Sigma) on the stainless steel sample plate and air-dried prior to analysis. External calibration was performed with enolase (Baker's yeast, Sigma) using the *m/z* value of 46,672 Da for the mono-charged ion and 23,336 Da for the dicharged ion. The values expressed are average mass and correspond to the $[M+H]^+$ ion.

2.7. N-terminal amino acid sequence analysis

The fractions from hydroxyapatite column were identified by Edman degradation, performed on an Applied Biosystems (Foster City, CA) gas-phase Sequencer model 492. A model 140 HPLC system (Applied Biosystems) and a model 785A Absorbance detector were used to identify the derivatized amino acid removed at each sequencing cycle. Retention times and integration values of peaks were compared to the chromatographic profile obtained for a standard mixture of derivatized amino acids (Applied Biosystems PTH standard kit). The procedure and reagents used were as recommended by the manufacturer.

3. Results

3.1. Standard preparations of human plasma PON1 are generally insufficiently purified

Human plasma PON1 used in our previous studies was purified to homogeneity as determined by Coomassie Blue staining after SDS-PAGE (Fig. 1), according to the standard protocol adopted by almost all PON1 investigators [9]. This protocol was assumed to provide PON1 pure at ≥95%. A major band (~44 kDa) and a minor one (~39 kDa) were both considered to be PON1 monomer due to their reactivity with an anti-human PON1 monoclonal antibody. This micro

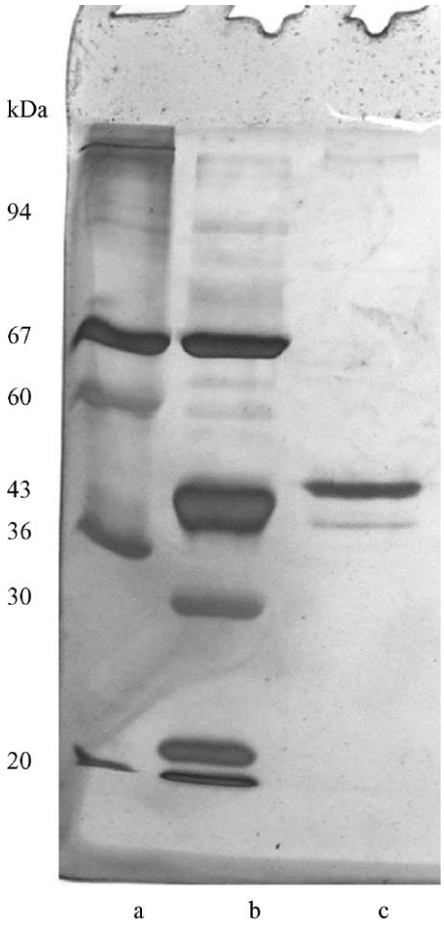

Fig. 1. SDS-PAGE analysis ($T = 10\%$) of a fraction of human plasma PON1 purified according to the standard protocol: lanes (a) and (b), molecular mass standards; lane (c), purified PON1. The major band (~44 kDa) and the minor one (~39 kDa) were both considered to be PON1 due to their reactivity with an anti-human PON1 monoclonal antibody.

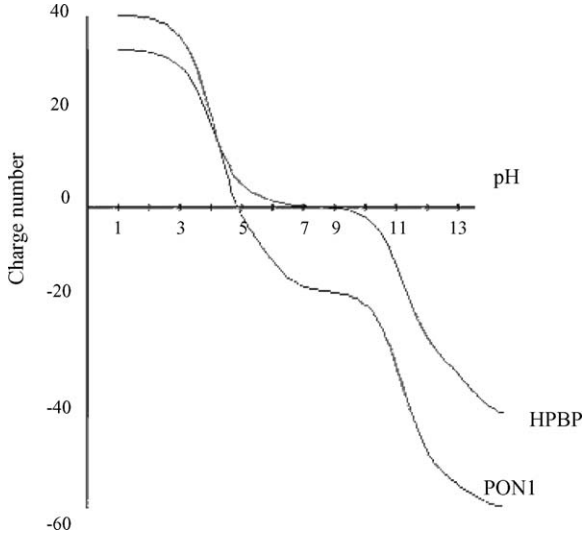

Fig. 2. Superimposition of theoretical titration curves of PON1 and HPBP. Curves were built using amino acid sequence data obtained by SwissProt protein data bank for PON1 (accession number P27169) and by crystallographic sequence, confirmed at >75% by classical sequencing [13] for HPBP.

heterogeneity has been currently attributed to glycoforms, the major band being regarded as native PON1, the minor one as a differently glycosylated form (human PON1 has three potential *N*-glycosylation sites). Unfortunately, the presence of this component systematically hindered detection of HPBP (~39 kDa). A batch of human PON1 purified according to these criterions led to diffractable crystals of HPBP [11,12]. N-terminal amino acid sequence determination was performed afterwards on crystal supernatant and re-dissolved crystal. The supernatant provided two sequences, AKLIALTLLGMGLALFR, corresponding to PON1, and XINGGGATLPQKLYLTP, showing no similarity to any described protein. The crystal provided the unknown sequence XINGGGATLPQ, only, i.e., HPBP. These results confirmed that the PON1 preparation contained an unknown contaminant, and that only this contaminant provided crystals. The unknown contaminant was later shown to be a human plasma protein capable to bind phosphate and subsequently termed human phosphate binding protein (HPBP) [13]. We thus reconsidered the purification protocol and the homogeneity of the purified PON1.

3.2. HPBP is strongly associated to PON1 in HDL particle and is co-purified

Due to the presence of crystallisable HPBP in PON1 samples, we focused on the quantitative occurrence of HPBP at the final step of the current purification protocol of PON1, i.e., the anion exchange chromatography step. Indeed, efforts to separate PON1 from HPBP using DEAE chromatography performed at pH 8.0, were intended because both proteins display non-overlapping theoretical titration curves above pH 5 (Fig. 2). Unfortunately, HPBP was always co-purified with PON1, and only harsh dissociating conditions (9.8 M urea and 4% Triton X-100) led to separation of HPBP from PON1 using 2D-PAGE [13]. This suggests that PON1 and HPBP are tightly associated. The similar MW of both proteins, and the fact that purity control of PON1 was currently performed by SDS-PAGE, explain why HPBP escaped to detection.

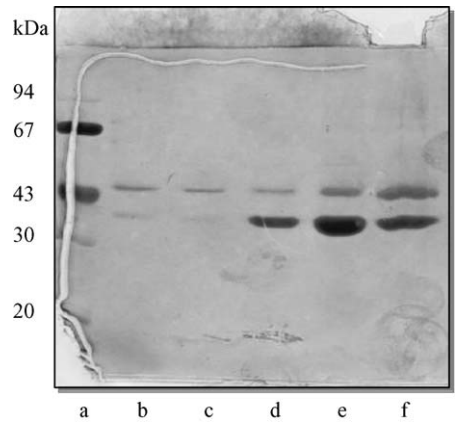

Fig. 3. SDS-PAGE ($T = 10\%$) of PON1 fractions: lane (a), molecular weight standards; lanes (b)–(f), PON1-containing fractions at the ion-exchange terminal step of standard protocol, issued from plasma bags of different individuals. The ratio PON1/HPBP varies considerably.

3.3. Hydroxyapatite chromatography allows tandem purification of PON1 and HPBP

Because human PON1 is a promising catalytic OP-scavenger, it is of importance to determine whether PON1–HPBP association is physiologically, pharmacologically and toxicologically relevant. Meanwhile, a prerequisite for taking on this study is the characterization of both proteins. The availability of purified PON1 and HPBP is thus a crucial issue. Due to the fact that the ratio PON1/HPBP varies considerably within plasma bags because of the lipoprotein status of blood donors (Fig. 3), we looked for a specific step allowing to separate PON1 and HPBP. We selected hydroxyapatite chromatography for the following reasons: (a) it was successfully used for purification of PON1 from rat liver since 1993 [19]; (b) the Ca^{2+}-dependence of PON1 and the PO_4^{2-}-binding property of HPBP suggested that hydroxyapatite chromatography could fulfil this purpose; (c) the fact that hydroxyapatite has been used for purification of membrane proteins, because the matrix works in the presence of detergents, was an additional argument. Indeed, although

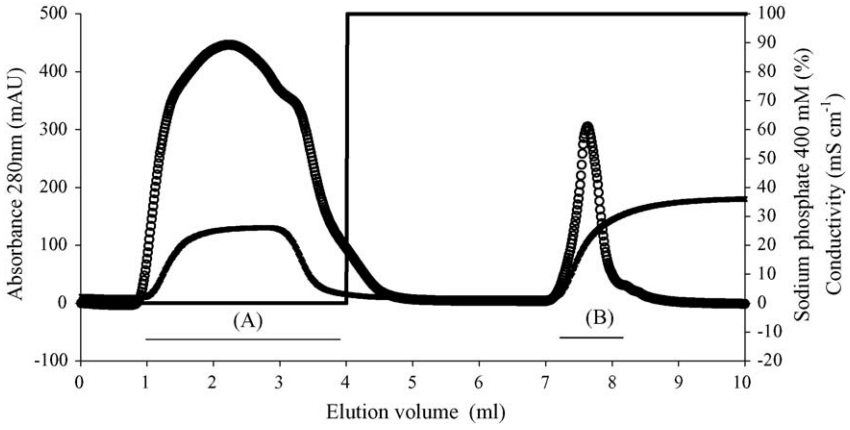

Fig. 4. Elution profile of HPBP and PON1 from Bio-Gel HTP hydoxyapatite media. Carrier, 10 mM sodium phosphate buffer, pH 7.0 (buffer A); displacer, sodium phosphate in buffer (A). Circles, UV absorbance at 280 nm; solid line, 400 mM sodium phosphate (%); dashed line, conductivity (mS cm^{-1}). UV absorbance of unbound material (peak A) is overestimated due to the presence of Triton.

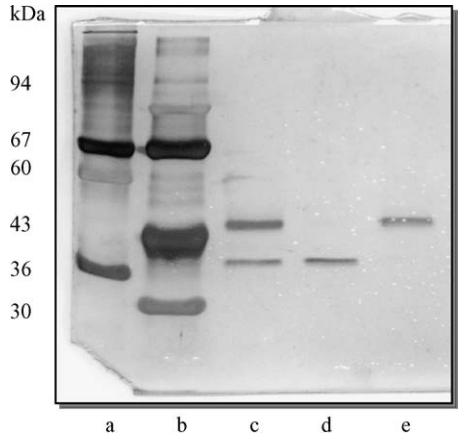

Fig. 5. SDS-PAGE analysis ($T = 10\%$) of PON1–HPBP fractions after hydroxyapatite chromatography. Lanes (a) and (b), molecular weight standards; lane (c), a fraction from the Q-Sepharose terminal step of conventional protocol; lanes (d) and (e), peak A and peak B containing purified HPBP and PON1, respectively.

PON1 is a soluble protein, after partial purification it remains stable only in a hydrophobic environment (i.e., in the presence of a detergent). Even though the exact mechanism of molecules separation on hydroxyapatite is not known, large amphoteric molecules such as proteins can be separated through differential interactions between matrix Ca^{2+} and PO_4^{2-} ions, and charged groups of biomolecule surface. Thus, fractions loaded on hydroxyapatite column contained 0.1% Triton X-100. Several batch conditions using different hydroxyapatite media were tested. After optimization of experimental conditions, injection of PON1/HPBP-containing sample in 25 mM Tris buffer containing 0.1% Triton X-100, on Bio-Gel HTP hydroxyapatite equilibrated with 10 mM sodium phosphate pH 7.0, followed by washing with the same buffer and elution by 400 mM sodium phosphate allowed to separate the two proteins (Fig. 4). HPBP was not retained on hydroxyapatite equilibrated without $CaCl_2$, and was collected in the filtrate. On the contrary, PON1 was retained and subsequently eluted by means of phosphate ions. Purity of each protein (PON1 and HPBP) was checked by SDS-PAGE (Fig. 5). The unadsorbed protein and the eluted protein provided a single band with a molecular mass corresponding to ~39 and ~44 kDa, respectively. Purity of each protein was subsequently confirmed by N-terminal sequencing and MALDI-TOF analysis, providing only a single sequence and one peak for each sample: DINGGGATLP and 38616.76 (m/z), corresponding to HPBP, AKLIALTLL and 44071.97 (m/z) corresponding to PON1.

4. Discussion

Because PON1 and HPBP are HDL-associated proteins, their relation with these particles have to be re-examined. A small subset of HDL particles having a complex dynamic structure is the predominant, physiological PON1 acceptor in plasma. Several proteins can be in some way associated to PON-bearing HDL, in addition to ApoA-I, their principal resident apolipoprotein component (Fig. 6). To remove contamination of platelet-activating factor acetylhydrolase (PAF-AH), and lecithin cholesterol acetyl transferase (LCAT), affinity chromatography on Concanavalin-A was used [20]. Other HDL-associated proteins such as apolipoproteins C, D, E, J, PAF-AH, and ghrelin were described as susceptible to contaminate PON1 samples. Trace amounts of PAF-AH were shown to be responsible for the PAF-acetylhydrolase activity attributed to PON1 [21]. Ghrelin, a circulating brain-gut peptide, was shown to bind a species of HDL associated with PON1, ApoA-I and ApoJ [22].

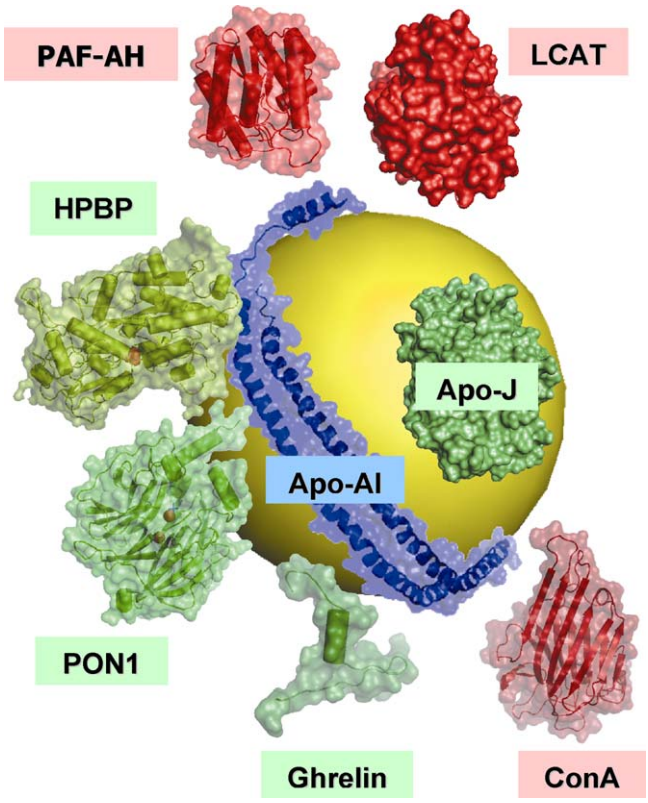

Fig. 6. Scaled schematic illustration for a PON1-containing HDL particle with durably or transiently associated proteins (ApoA-I, PON1, HPBP, Ghrelin, *ApoJ*), transiently co-localized proteins (PAF-AH, *LCAT*) proteins, or reagent products (Con A), having a propensity to contaminate purified PON1 fractions. Unsolved 3D structures are simulated. The HDL particle is a ~10 nm-diameter sphere with a non polar core of cholesteryl-ester and triglyceride molecules encapsulated by a surface monolayer of amphipathic α-helical apolipoprotein and phospholipid molecules.

The presence of HPBP in preparations of PON1 escaped to detection for a long time. This was due to the similar MW of both proteins and because purity was usually checked by SDS-PAGE. A 39 kDa protein generally observed was exclusively attributed to the presence of a deglycosylated form of PON1. We developed a 2D-PAGE technique, working under drastic dissociating conditions to provide evidence for the presence of HPBP in PON1 preparations. Interestingly, 2D-PAGE data reported in previous studies are in agreement with our results. In the first one, a spot in the stained gel showed that a protein having a MW and a p*I* corresponding to HPBP co-purified with PON1 [23]. In the second one, only PON1 was revealed by silver staining and Western blot [24], but the PON1 sample came from electro-elution of a band excised from a SDS-PAGE. Thus, we

assume that HPBP is associated with PON1 in vivo and remains so along purification steps. HPBP escaped to detection and/or was ignored for two main reasons: (1) the protein was not beforehand described; (2) a gene sequence corresponding to HPBP has not been found in either the human genome or EST databases. HPBP is related to a family of still poorly described proteins, named DING according to the sequence of their four conserved N-terminal residues. Finally, we showed that the novel human HDL-associated apolipoprotein, HPBP, belongs to the family of ubiquitous eukaryotic proteins named DING [13].

Hydroxyapatite chromatography has previously been successfully used as the first step for purification of microsomal rat liver PON1 [19,25] and PON3 [26], rabbit liver microsomal PON1 [27]. Besides, it was also used as the final step to purify variants of mammalian recombinant PON1 (issued from a synthetic construct from rabbit, mouse, rat, and human PON1 genes) expressed in *E. coli* [7]. As far as we know, hydroxyapatite chromatography was never used for plasma PONs, thus, the behaviour of the couple PON1–HPBP towards hydroxyapatite looked at first unforeseeable. Basic proteins are not supposed to be retained on hydroxyapatite columns equilibrated with Ca^{2+}, while acidic proteins are supposed to be eluted with phosphate buffers of low molarity (<100 mM). However, theoretical titration curve (Fig. 2), 2D-PAGE [13] and capillary electrophoresis analysis (Rochu et al., in preparation) suggest that HPBP is neutral in the pH range 7–9. A high isoelectric point could explain the non-binding of HPBP to hydroxyapatite matrix. By contrast, PON1 contains calcium ions and is negatively charged in the same pH range. In addition, there is a phosphate ion bound to the catalytic calcium of recombinant PON1 [14]. These features may explain the binding of PON1 to the matrix. In other respects, a strong association between PON1 and HPBP is assumed. Thus, an additional mechanism could be the competition of the calcium phosphate matrix with the protein binding regions of the two apolipoproteins forming the PON1–HPBP complex. As it was suggested, PON1 displays a canopy that controls access of the active site and is also involved in anchoring PON1 to the HDL particle [14]. Our results provide new evidence that for hydroxyapatite chromatography, adsorption and elution of proteins is the reverse of a simple process. Interaction of proteins with hydroxylated calcium phosphate [$Ca_5(PO_4)_3(OH)_2$] forming both a matrix and a ligand obeys different mechanisms. In our attempt at obtaining free-PON1, hydroxyapatite chromatography was at length a crucial purification step as it allowed to separate PON1 and HPBP in a single step.

5. Conclusion

The hydroxyapatite chromatography step described here is at the moment the only one offering high-resolution tandem separation of PON1 and HPBP. For HPBP, there was up to now no purification protocol. This should stimulate studies of the first inorganic phosphate transporter so far reported in human plasma. Besides, our results corroborate current literature data indicating that certain activities and stability of PON1 depend decisively on the enzyme molecular environment. These functional distinctions could be attributed to the absence or presence of variable amounts of HPBP. Highly purified PON1 is now intended for study of its functional stability and activity in the absence of its partner. Because PON1 is a promising catalytic OP scavenger to be used in prophylaxis and/or treatment of OP poisoning, it will be important to develop media that mimic in vivo conditions. To date, very few kinetic studies on plasma PON1 have been carried out under 'physiological conditions'. HDL particles have a fairly short half-life (2–2.5 days) which may preclude a major impact of inactivation on PON1 during the residence of the enzyme in blood [28]. Since 'natural' HDL environment of PON1 is reputed as inconstant, knowledge of partner lipoprotein(s) or/and hydrophobic cofactor(s) able to act as surrogate stabilizing environment for active PON1 is essential.

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