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Lipid complex of apolipoprotein A-I mimetic peptide 4F is a novel platform for Paraoxonase-1 binding and enhancing its activity and stability

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Abstract

High density lipoprotein (HDL) associated paraoxonase-1 (PON1) is crucial for the anti-oxidant, anti-inflammatory, and anti-atherogenic properties of HDL. Discoidal apolipoprotein (apo)A-I:1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) complex has been shown to be the most effective in binding PON1, stabilizing it, and enhancing its lactonase and inhibitory activity of low density lipoprotein oxidation. Based on our earlier study demonstrating that apoA-I mimetic peptide 4F forms discoidal complex with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, we hypothesized that lipid complexes of 4F would be able to bind PON1 and enhance its activity and stability. To test our hypothesis, we have expressed and purified a recombinant PON1 (rPON1) and studied its interaction with 4F:POPC complex. Our studies show significant increase, compared to the control, in the paraoxonase activity and stability of rPON1 in the presence of 4F:POPC complex. We propose that 4F:POPC complex is a novel platform for PON1 binding, increasing its stability, and enhancing its enzyme activity. We propose a structural model for the 4F:POPC:PON1 ternary complex that is consistent with our results and published observations.

Keywords

High density lipoprotein; Peptide; Amphipathic helix; Apolipoprotein A-I; Lipid; Paraoxonase-1

Introduction

In the general population plasma low density lipoprotein (LDL) cholesterol exacerbates atherosclerosis whereas high density lipoproteins (HDL) cholesterol appear protective, i.e. HDL cholesterol levels are inversely related with the risk of cardiovascular disease (CVD)

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[1]. However, in people with high CVD risk a substantial residual risk remains even after reducing LDL levels. This emphasizes the need for additional new therapeutic approaches against CVD; a pharmacological intervention that increases the level of cardioprotective functional HDL is a potential solution. Evidence suggests that the cardiovascular benefits of HDL are realized because of its anti-oxidant, anti-inflammatory, and anti-atherogenic properties. All of these beneficial effects depend on an HDL-associated enzyme called paraoxonase-1 (PON1). Indeed, PON1 inhibits LDL oxidation and this enzymatic activity inversely correlates with CVD risk [2].

Reduced PON1 expression in animal models is associated with increased formation of atherosclerotic lesions, increased oxidative stress, and reduced antioxidant capacity of HDL [3; 4]. Recent prospective studies have provided compelling evidence for PON1 also being an independent cardiovascular risk factor in humans [5; 6]. An important determinant of PON1 activity is HDL. HDL is the transport vehicle for PON1 in plasma. It has been demonstrated in several *in vitro* and *in vivo* studies that the anti-inflammatory capacity of HDL is reduced when the PON1 content of HDL is reduced [7]. There is growing evidence that the anti-oxidant and anti-inflammatory capacity of HDL is a feature of its beneficial impact on vascular health and, correspondingly, reductions in this capacity contribute to “dysfunctional HDL” and increased vascular risk [8]. PON1 has recently been identified as a key factor for the bioactivation and clinical activity of antiplatelet drug clopidogrel [9]. Pathological conditions associated with oxidative stress, such as chronic renal failure, rheumatoid arthritis and Alzheimer’s disease, are frequently associated with reduced activity of PON1 [10]. Considering the growing evidence of the protective role of PON1 in coronary artery disease, this enzyme represents an important target for pharmacological regulation [11].

Human PON1 can hydrolyze the toxic oxon metabolites of a number of insecticides such as parathion, diazinon and chlorpyrifos, and even nerve agents such as sarin and soman [12]. The presence of PON1 in the blood, liver and some other tissues should provide protection against chronic exposure to low levels of organophosphates, and has the advantage that the enzyme acts catalytically, rather than binding them stoichiometrically [13]. It has been demonstrated recently that purified human and rabbit serum PON1 significantly protect against sarin and soman exposure in guinea pigs, supporting the development of PON1 as a catalytic bioscavenger for protection against lethal exposure to chemical warfare nerve agents [14].

Human PON1 is synthesized in the liver and secreted into the blood, where it associates with HDL. The exact mechanisms of HDL loading/unloading are unknown, but binding of PON1 to HDL is known to stabilize PON1 and increase its enzymatic and antioxidant activities [15]. Furthermore, among the apolipoproteins examined (apoA-I, apoA-II, apoA-IV, apoE3 and apoE4), discoidal apoA-I:POPC complexes have been shown to be the most effective in binding PON1, stabilizing it, and enhancing its lactonase and inhibitory activity of LDL oxidation [15; 16].

Human apoA-I contains 243 amino acid residues, but much shorter peptides that we designed and studied in our laboratory have been shown to mimic many of the physical-chemical and biological properties of full-length apoA-I [17–19]. Using high-resolution solution NMR, we have determined structures of apoA-I mimetic peptides 2F and 4F in discoidal complexes of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) [18; 19]. Based on our structural studies demonstrating that apoA-I mimetic peptide 4F forms discoidal complexes with lipid [19], we hypothesized that lipid complexes of 4F would be able to bind PON1, increase its stability, and improve its activity. To test our hypotheses, a recombinant PON1 (rPON1; clone G3C9, kindly provided by Dr. Dan S. Tawfik, Weizmann

Institute of Science, Israel) was expressed in *Escherichia coli* and purified. Our studies have shown significant increase, compared to the control, in the paraoxonase activity and stability of rPON1 in the presence of 4F:POPC complex. Based on these exciting observations, we propose that lipid complexes of apoA-I mimetic peptides are novel platforms for PON1 binding, increasing its stability, and enhancing its activity. These studies on rPON1 have the potential for setting a paradigm for undertaking similar studies of other HDL-associated proteins and enzymes in their native-like environment. In addition, these studies may lead to the development of novel formulations of rPON1 for therapeutic use.

Materials and Methods

Peptide synthesis, purification, and lipid complex preparation

The peptide 4F (Ac-DWFKAFYDKVAEKFEAF-NH₂) was synthesized and purified as described earlier [20]. POPC was obtained from Avanti Polar-Lipids, Inc., Alabaster, AL. 4F:POPC complex was prepared essentially as described by us earlier [19].

Expression and purification of recombinant PON1 (rPON1)

We have used clone G3C9, kindly provided by Dr. Dan S. Tawfik, Weizmann Institute of Science, Israel, to express and purify rPON1. G3C9 carries an eight-Histidine tag on the C-terminus that has no effect on PON1's enzymatic activity and HDL binding [16]. The clone G3C9 is a very close homologue of wild-type rabbit PON1 (95% amino acid identity and 98% similarity) and, in detergent micellar solution, was shown to possess enzyme properties identical to those of rabbit as well as human PON1 with a wide range of substrates [21]. It has been shown that G3C9 preserves both the enzyme properties of native PON1 and its HDL binding properties, thus making it a useful tool in the mechanistic and structural studies of PON1 [15]. BLAST analysis (<http://blast.ncbi.nlm.nih.gov>) reveals 86% identity and 93% similarity between human PON1 and rPON1 (clone G3C9). rPON1 was expressed and purified essentially as described [21].

Binding of rPON1 with 4F:POPC complex

The binding of rPON1 to 4F:POPC complex was analyzed by ELISA, essentially as described by Gaidukov and co-workers [16]. Briefly, 96-well plates (Costar) were coated with 10 µg/mL of the complex by incubating them for 3 hrs at room temperature. The wells were blocked with 1% BSA (Sigma; A7030-50G, lot 110M1661V) by incubating at room temperature for 1 hr. After washing the plate with PBS, rPON1 was added at increasing concentrations. The plates were incubated at room temperature for 3 hrs. After the plates had been washed with PBS, a monoclonal anti-PON1 antibody (obtained from the Hybridoma Core Facility, University of Michigan) was added at 1:1600 dilution in 0.1% BSA and the plates were incubated at room temperature for 1 hr. The monoclonal antibody was purified from the ascites by immunoaffinity chromatography using fast-flow protein A-Sepharose (GE Healthcare). The specificity of the antibody was confirmed using human plasma obtained from the American Red Cross. After washing the plates with PBS, a secondary antibody (Goat pAb to Mouse IgG (HRP); Abcam, ab6789; lot GR23382-1) at a 1:2500 dilution in 0.1% BSA was added. After 1 hr incubation at room temperature and washing with PBS, plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB; Thermo Scientific; 34028, lot MB1375812)) as substrate. The reaction was stopped by addition of 1 M H₂SO₄, and the optical density was measured at 450 nm.

Preparation of rPON1-containing 4F:POPC

rPON1 was added to 4F:POPC complex at a ratio (wt:wt) of 1:100 in 100 mM TRIS, pH 8.0, buffer containing 2 mM calcium chloride (PON buffer). This ratio was selected based

on the results of our binding experiment as described above. After 3 hrs incubation at 37°C, the complex was purified using size exclusion chromatography on a FPLC system (BioLogic, DuoFlow, BIO-RAD). This system uses Superose 6 10/300 GL and Superdex 200 10/300 GL columns (Amersham Biosciences/GE Healthcare) linked in tandem and run at a flow rate of 0.4 mL/min in PBS containing 0.02% sodium azide (pH 7.4) as described earlier by us [18; 19]. Elution was monitored using UV absorption at 280 nm. The fractions were collected and analyzed for enzyme activity and the presence of rPON1, peptide, and POPC. rPON1 was detected using SDS-PAGE followed by Western blotting using PON1 antibody.

The amounts of lipid and peptide in the eluted fractions were measured using an enzymatic colorimetric method (Phospholipids B, Wako Chemicals USA, Inc.) and absorbance at 280 nm (using an extinction coefficient of $7300 \text{ M}^{-1}\text{cm}^{-1}$) in the presence of 8M guanidinium hydrochloride, respectively.

Paraoxonase activity in the FPLC fractions were determined essentially as described by Gan and co-workers [22]. Briefly, paraoxonase activity was measured by UV spectrophotometry in a 96-well plate format (BIO-TEK Synergy HT multi-detector microplate reader) using 1.0 mM paraoxon (Sigma-Aldrich, St Louis, Mo) as a substrate. Activity was measured in 100 mM Tris/HCl buffer at pH 8.0 containing 1 mM calcium chloride at 25°C. The amount of p-nitrophenol was calculated from the molar extinction coefficients (412 nm) at pH 8.0 of $17,100 \text{ M}^{-1} \text{ cm}^{-1}$.

Trypsin digestion of rPON1 in the absence and presence of 4F:POPC complex

Sequencing grade trypsin was obtained from Promega (Promega Corporation, Madison, WI). rPON1 was digested with trypsin using rPON1:trypsin ratio of 100:1 (wt:wt). The mixture was incubated at 37°C for 0, 15, 30, 60, 120, 180, 240, 300, 360 minutes. 100 μL aliquotes were taken out at these time points and were frozen for SDS-PAGE analysis.

Molecular modeling

Molecular modeling was performed using the program SYBYL (version 7.3) (Tripos, Inc., St. Louis, MO). We have modeled the structure of human PON1 based on the crystal structure of a variant of PON1 (clone G2E6) (protein data bank identification code 1V04) [23] using SWISS-MODEL, a homology based protein modeling software [24].

Results and Discussion

HDL represents a heterogeneous mixture of lipoproteins with hydrated densities in the range from 1.063 to 1.210 g/mL and diameters in the range from 5–14 nm [25]. HDL-associated PON1 is crucial for the anti-oxidative, anti-inflammatory, and anti-atherogenic properties of HDL. PON1 has been shown to associate exclusively with small, dense HDL particles (HDL3c; density range, 1.155–1.170 g/ml, average diameter 7.3 nm) that are most effective in preventing LDL oxidation [26]. In addition to PON1, a number of other proteins, including apoA-I, have been identified in these particles [27]. Among the apolipoproteins examined (apoA-I, apoA-II, apoA-IV, apoE3 and apoE4), discoidal apoA-I:POPC complexes have been shown to be most effective in binding PON1, stabilizing it, and enhancing its lactonase and inhibitory activity of low density lipoprotein oxidation [15; 16]. Based on the results obtained in these studies, it has been suggested that PON1:HDL recognition is based primarily on surface properties of the apolipoproteins and that specific protein-protein interactions may play only a secondary role [16].

Figure 1A shows binding of rPON1 with 4F:POPC complex. In this experiment, increasing amounts of rPON1 were added to a constant concentration of 4F:POPC complex. Figure 1B

shows increase in the paraoxonase activity of rPON1 (used at a constant concentration) in the presence of increasing concentrations of 4F:POPC complex. It is evident that rPON1 binds to 4F:POPC complex (Figure 1A) and binding is accompanied by an increase in the paraoxonase activity of rPON1 (Figure 1B). Thus, rPON1 binds to 4F:POPC in its native-like structure since its enzyme activity is not only preserved but also enhanced. When compared on a weight basis, similar binding and paraoxonase activity of rPON1 was observed in the presence of 4F:POPC and apoA-I:POPC complexes (results not shown).

Figure 2A shows size exclusion elution profiles of 4F:POPC complex in the absence and presence of rPON1. Figure 2B shows the results of native PAGE (4–20%) of the FPLC fractions 14 through 17 (fractions corresponding to the elution peak) of the 4F:POPC complex in the absence and presence of rPON1. Both size exclusion chromatography (Figure 2A) and native PAGE (Figure 2B) indicate that there is no apparent change in the size of the 4F:POPC complex in the presence of rPON1. FPLC fractions 14–17 were further analyzed for the presence of rPON1 and 4F using reducing SDS-PAGE (4–20%). The results are shown in Figure 2C. It is evident that fractions 14–16 contain rPON1 (Figure 2C). To further confirm rPON1 in the FPLC fractions, Western Blot analysis was performed on the fractions 11–19. The results are shown in Figure 2D. It is evident that fractions 12 through 16 contain rPON1, with higher amounts of rPON1 being present in fractions 14 and 15.

Figure 3 shows the results of trypsin digestion of rPON1 in the presence and absence of 4F:POPC complex. It is evident that presence of 4F:POPC stabilizes rPON1 against trypsin digestion (Figure 3).

With a view to establish a high-resolution method to understand subtle differences in peptide:lipid complexes, we have used 2D ^1H -NMR spectroscopy to investigate the structure of amphipathic helical peptides, including 4F, bound to DMPC disc [18; 19]. These studies for the first time showed that the peptide helix axis is oriented parallel to the bilayer plane on the edge of the disc [18; 19]. Based on the results of chemical cross-linking of the Lys residues in the peptides on the disc and molecular modeling, we proposed that amphipathic a helices arranged in an antiparallel fashion, cover the lipid acyl chains on the edge of the disc [18; 19].

Because of its anti-atherosclerotic properties, and because of its ability to detoxify nerve agents that are both a terrorist threat and a nonconventional military weapon, PON1 is currently the subject of intense research. Despite a large number of studies indicating biological importance of PON1, there is very little information available regarding its structure in HDL and its molecular interactions (with lipid and other HDL-associated proteins) in HDL. Based on the crystal structure of a PON1 variant a model of association of PON1 with HDL has been proposed [28]. However, the N-terminal residues 1–15 of PON1 in the crystal structure were not visible, and, therefore, their role in the association of PON1 with HDL is not known. It is important to note that these residues are part of the hydrophobic signal sequence of PON1 that has been shown to be important for its association with HDL [29]. In the crystal structure, the N-terminal hydrophilic part, which extends beyond the signal peptide (residues 19–28), adopts a helical structure (H1). Helix H2, adjacent to H1, is amphipathic. Helices H1 and H2 form two adjacent hydrophobic patches that provide a potential membrane-binding surface. In the crystal structure, the interface with HDL was further defined by a characteristic ‘aromatic belt’ rich in tryptophan and tyrosine side chains, and by a Lys side chain on H1 [30].

Recently, a theoretical model has been proposed for the association of PON1 with apoA-I-containing reconstituted discoidal HDL particles [31]. This model proposes interaction

between apoA-I, mediated through its Arginine residues located on the right side of the polar face of the amphipathic helices, and aromatic residues in PON1 [32]. This model, however, awaits experimental support. We reported previously that Arg analogs of 4F have higher paraoxonase activity than 4F [17]. However, this difference is presumably related to the differences in the structural properties of their POPC complexes [17].

Although it has been demonstrated that the signal sequence is important for the association of PON1 with HDL [33], its location, i.e., whether bound on the surface or buried in the hydrophobic interior of the HDL particles, is unknown. An examination of the amino acid composition of the signal sequence of human PON1 reveals that, in addition to aliphatic (e.g., Leucine and Isoleucine) nonpolar residues, it contains positively charged (Lysine and Arginine) as well as aromatic (Phenylalanine, Tyrosine, and Histidine) residues (Figure 4). Based on our studies of lipid-associating properties of model peptides containing charged and aromatic amino acid residues, we hypothesize that the signal sequence in PON1 that contains both charged and aromatic amino acid residues, and the amphipathic helix 2 identified in the crystal structure of variant PON1 [34], allow binding of PON1 to the face of the 4F:POPC complex as shown in Figure 4.

The proposed model for the 4F:POPC:PON1 ternary complex (Figure 4) is consistent with the following observations. There is no apparent change in the size of the 4F:POPC complex in the presence of rPON1 (Figure 2). Binding of rPON1 to the edge of the disc is likely to result in the increased hydrodynamic diameter of the disc. Furthermore, it has been proposed that HDL acquires PON1 by desorption from the hepatic cell membrane in a process mediated or facilitated by transient anchoring of HDL to the membrane [35]. This implies transfer of PON1 from the cell membrane to the cell membrane-like outer shell of the lipoprotein. The reverse process, i.e., transfer of PON1 from HDL to cell membranes, demonstrating the ability of PON1 to operate outside its HDL transport vector, has also been examined recently [36]. It has been shown that PON1 is not a fixed component of HDL, suggesting that the enzyme could also exert its protective functions outside the lipoprotein environment; these observations may be of relevance to tissues exposed to oxidative stress and/or bacterial infection [37]. Thus, these observations are consistent with the “exchangeable” nature of HDL-associated PON1. Burial of the PON1 signal sequence in the hydrophobic interior of HDL particles, as has been suggested previously [38], is likely to hinder transfer of PON1 from HDL to cell surfaces.

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The abbreviations used are

apoA-I	apolipoprotein A-I
CVD	cardiovascular disease
ELISA	enzyme linked immunosorbent assay
FPLC	fast protein liquid chromatography
HDL	high density lipoprotein
LDL	low density lipoprotein

PON1 paraoxonase-1
rPON1 recombinant PON1 (clone G3C9)

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Highlights

- Paraoxonase-1 imparts anti-atherogenic properties to high density lipoprotein.
- 4F is an apolipoprotein A-I mimetic peptide.
- Similar to apolipoprotein A-I, 4F forms discoidal lipid complexes.
- Lipid complex of 4F is a novel platform for Paraoxonase-1 binding.

FIGURE 1. Interaction of rPON1 with 4F:POPC complex

(A). Binding of rPON1 to 4F:POPC complex determined using ELISA. Increasing amounts of rPON1 was allowed to bind to a fixed concentration of 4F:POPC complex. (B). Increase in the paraoxonase activity of rPON1 in the presence of increasing amounts of 4F:POPC complex. Experiments were performed in triplicate and the error bars are standard error of mean.

FIGURE 2. Characterization of 4F:POPC complex in the absence and presence of rPON1
 (A) Size exclusion chromatography (Superdex 200 10/300 GL column) of 4F:POPC (red line) and 4F:POPC:rPON1 (black line). (B) Native PAGE (4–20%) analysis of FPLC fractions 14–17 of 4F:POPC complex in the absence (lanes 2–5) and presence (lanes 7–10) of rPON1. (C). Reducing SDS-PAGE (4–20%) analysis of FPLC fractions 14–17 of 4F:POPC complex in the presence (lanes 2–5) and absence (lanes 7–10) of rPON1. Lane 1 is molecular shows molecular weight standards and lane 2 shows purified rPON1 (5 μ g). (D) Western blot analysis of FPLC fractions (11 through 19) of 4F:POPC obtained in the presence of rPON1.

FIGURE 3. Stability of rPON1 in the absence and presence of 4F:POPC complex

Trypsin digestion of rPON1 (uncomplexed, black bars; complexed with 4F:POPC, red bars). Digestion was carried out in 50 mM Tris buffer, pH 8.0, at 37°C, with an enzyme to rPON1 ratio of 1:100 (wt:wt). rPON1 was quantified by Image J analysis of Coomassie Blue stained 4–20% SDS-PAGE of rPON1. The experiments were performed in triplicate and error bars represent standard error of mean.

FIGURE 4. A molecular model of 4F:POPC:PON1 ternary complex

A proposed model showing mode of association of human PON1 (shown in the cartoon representation) with 4F:POPC complex (shown in the space filled representation). In PON1 structure, H1 represents helix 1 and H2 represents helix 2. The entire signal sequence of human PON1 (H1) was modeled as an α -helix. Aromatic and charged residues in H1 and H2 are shown in space filled representation (Lys and Arg, blue; Phe, green; Tyr, yellow; Trp, gold; His, magenta). 4F molecules are shown to shield the nonpolar edge of the lipid bilayer of the discoidal particle. We propose that rPON1 associates with 4F:POPC discoidal complex through its signal sequence (helix 1, H1) and amphipathic helix 2 (H2) binding to the face of the disc.