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Crystallization and Preliminary X-ray Crystallographic Analysis of Human Plasma Platelet Activating Factor Acetylhydrolase

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Abstract

The plasma form of the human enzyme platelet activating factor acetylhydrolase (PAF-AH) has been crystallized, and X-ray diffraction data were collected at a synchrotron source to a resolution of 1.47 Å. The crystals belong to space group *C2*, with unit cell parameters of $a = 116.18$, $b = 83.06$, $c = 96.71$ Å, and $\beta = 115.09^\circ$ and two molecules in the asymmetric unit. PAF-AH functions as a general anti-inflammatory scavenger by reducing the levels of the signaling molecule PAF. Additionally, the LDL bound enzyme has been linked to atherosclerosis due to its hydrolytic activities of pro-inflammatory agents, such as *sn*-2 oxidatively fragmented phospholipids.

Keywords

PAF-AH; lipoprotein associated phospholipase A2; Lp-PLA2; group VIIA PLA2; crystallization

INTRODUCTION

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid-signaling molecule that plays an important role in normal physiological processes of the body, as well as an inflammatory mediator in pathological processes [1]. The enzyme PAF-AH was first identified from the plasma by its ability to inactivate PAF [2]. The enzyme PAF-AH has been classified as a group VIIA phospholipase A2 (PLA2) enzyme [3], and as such it hydrolyzes the ester bond at the *sn*-2 position of its phospholipid substrates. Another generally used name for plasma PAF-AH is lipoprotein associated PLA2 (Lp-PLA2) [4]. In addition to its role in reducing PAF levels, PAF-AH functions by hydrolyzing other pro-inflammatory agents, such as oxidized lipids of LDL particles [5]. Many of these oxidized phospholipids have an oxidatively fragmented *sn*-2 chain, analogous to the signaling molecule PAF.

In addition to the secreted plasma form of PAF-AH, a homologous (42% identical) intracellular form, referred to as type-II PAF-AH (also known as group-VIIB PLA2), is believed to perform similar functions to the plasma enzyme, but inside liver and kidney cells [6, 7]. Physiologically, these PAF-AH enzymes are associated with lipoproteins or the inner-leaflet of kidney and liver cells, and as such, are considered interfacial enzymes, which function on the lipid-aqueous interface. Structurally distinct from the 44 kDa plasma PAF-AH and type-II PAF-AH enzymes, is a 26 kDa intracellular brain catalytic domain that has been designated type-I PAF-AH, or more commonly PAF-AH-Ib [7]. Although the structure of PAF-AH-Ib has been reported [8, 9], there is no sequence homology to the other PAF-AH

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enzymes. Additionally, the PAFAH-Ib enzyme has been suggested to have a physiological function other than as a true PAF-AH enzyme [10, 11].

In contrast to the 14 kDa secreted PLA2s, the PAF-AH enzymes are calcium independent and contain a GX SXG motif that is characteristic of neutral lipases and serine esterases [12]. Based on their protein sequences and distant homology to other lipases, the PAF-AH enzyme structures are predicted to have a typical α/β -hydrolase fold of lipases and esterases. Although a structure has been determined for a distantly related lipase from *S. exfoliates* [13], the extent of sequence overlap between this enzyme and the mammalian PAF-AH enzymes is quite limited. Hence, a crystal structure of a mammalian PAF-AH is desirable.

Although the importance of plasma PAF-AH was first suggested in the 80's [2, 14], the cDNA encoding human pPAFAH was not cloned until 1995 [15]. The enzyme functions as a monomeric 43, 44, or 45 kDa protein. The amino terminus of the physiologically mature protein is heterogeneous with N-terminal starting residues of S35, I42 or K55 [12]. Following the enzyme's identification in the plasma, it had been subjected to extensive biochemical characterization, including the characterization of the limits of N- and C-terminal truncations that preserve native functions [12]. Here, we report the crystallization and preliminary X-ray diffraction of a 43.4 kDa construct (residues 47-429) of PAF-AH. This construct represents a form of the enzyme with native-like enzyme activity [12] toward the substrate PAF, as well as native-like LDL binding properties [16].

MATERIALS AND METHODS

Protein Preparation

Through collaboration with ICOS Corporation we have obtained 160 mg of pure human plasma PAF-AH (residues 47-429) that was overexpressed from *E. coli*. The construct's start at residue-47 is within the range of the natural heterogeneity observed in its N-terminal start positions, as discussed above. The construct's C-terminus at residue-429 corresponds to a C-terminal truncation of 12 residues compared to the coding region of human plasma PAF-AH (NCBI accession Q13093). A thorough characterization of this construct of plasma PAF-AH [12, 16, 17] had demonstrated that this truncation of the protein is fully functional. Details of the *E. coli* expression and purification of this construct of plasma PAF-AH were previously reported [18]. The PAF-AH enzyme used in our studies was provided by ICOS Corp., and it had been lyophilized from a detergent/buffer solution (ICOS Corp. formulation buffer) with PAF-AH originally at 10 mg/ml and containing 15 mM sodium citrate, 7.5% (w/v) sucrose, 0.1% (w/v) Pluronic F68, 0.05% (w/v) Tween 80, pH 6.5. Prior to experiments, samples were dissolved in H₂O or 10 mM Tris-HCl (pH 7.0) and assayed for protein concentration by BCA assay.

Initial protein crystallization efforts focused on PAF-AH enzyme that had been exchanged into a buffer that contained a single detergent component, Triton DF16. The buffer composition, detergents and protein concentration of PAF-AH were adjusted following the binding of PAF-AH to a 1 ml anion exchange Hi Trap Q-sepharose column equilibrated with 10 mM Tris-HCl, pH 7.0, 0.01% Triton DF16. The eluting buffer contained 20 mM Tris-HCl, pH 7.0, 1M NaCl and 0.01% Triton DF16. A gradient of zero to 1 M sodium chloride was used to elute the protein between 0.27 to 0.50 M sodium chloride. The eluted protein was then dialyzed against a solution of 10 mM Tris-HCl, pH 8.5, 0.01% Triton DF16 to remove the salt. Then protein was concentrated by centrifugal concentration using a centricon-30 membrane (Millipore) at slow speed (2000 rpm) to avoid aggregation and precipitation. PAF-AH was assayed for kinetic activity using the ester substrate *p*-nitrophenyl acetate, and the reaction was followed by UV absorbance at 402 nm ($\epsilon_{402\text{ nm}} = 17,700\text{ M}^{-1}\text{cm}^{-1}$).

Crystallization

Plasma PAF-AH was exchanged using a 25 kDa cutoff dialysis membrane (Spectrum) into a variety of buffer/detergent solutions containing 20 mM Tris, pH 7.5, 150 mM NaCl and a choice of several detergents, either alone or in combination. The detergents explored included CHAPS, Tween 20, Triton DF12, Triton DF16, and n-octyl- β -D-glucopyranoside (BOG) purchased from Sigma Aldrich Chem. Co. PAF-AH exchanged into a specific detergent composition was then screened using commercial screens from Hampton Research (screen-1, screen-2, PEG/ion, cryo, phosphate, and ammonium sulfate screens), which led to several promising conditions. Our initial protein crystals of plasma PAF-AH were obtained in the detergent Triton DF16 at 0.06 % (w/v). This detergent offers the advantage of having a CMC value in the high mM range, thereby allowing protein to be exchanged and concentrated at detergent concentrations below the detergent CMC. Additionally, under this condition the detergent was in great excess of the protein concentration. Following optimization of an initial hit from condition #34 of Hampton Research crystal screen-2, we obtained small protein crystals (<50 microns) that diffracted X-rays cleanly to a resolution of 3.1 Å at the synchrotron. The crystals were formed at 20 °C by hanging drop using a crystallization reservoir solution composed of 0.1 M HEPES, pH 7.7, 0.6 M sodium acetate, 0.05 M cadmium sulfate, 0.6 M sucrose, and 0.06 % (w/v) Triton DF16.

A second crystal form (>100 microns) was obtained using a combination of the detergents Triton DF16 (0.01 % w/v) and BOG (25 mM), and crystal screening was performed, as described above. Using this detergent combination, our best protein crystals were grown at 20 °C using a crystallization reservoir solution composed of 0.1 M MES, pH 6.0, 2.2 M (NH₄)₂SO₄, 0.01 % (w/v) Triton DF16 and 25 mM BOG. These crystals diffracted X-rays cleanly to a resolution of 4 Å on our home Rigaku RUH3R/Raxis IV X-ray instrument and scaled in the C2 space group. Following additional screening, we found that the addition of sucrose, DTT, and 1,4-butanediol improved the visual quality of crystals.

However, our best improvement in crystal quality came from combining what we learned from the screening described above with the additional components of the formulation buffer (40 % of full strength formulation buffer) which were present in the lyophilized samples of PAF-AH obtained from ICOS Corp. From this point on, we resuspended the lyophilized PAF-AH in 10 mM Tris-HCl (pH 7.0) to a concentration of 4 mg/ml. Our best protein crystals of PAF-AH were obtained at 20 °C starting from a protein solution at 4 mg/ml that contained 10 mM Tris-HCl, 6 mM sodium citrate, 3% (w/v) sucrose, 1.0 mM DTT, 27 mM BOG, 0.04% (w/v) Pluronic F68, 0.002% (w/v) Tween 80, pH 6.7. The crystallization reservoir solution contained 98.5 mM MOPS buffer, pH 6.6, 44.3% (w/v) (NH₄)₂SO₄, 0.394 M Li₂SO₄, 0.985 M sodium acetate, and 1.48% (v/v) 1,4-butanediol. Prior to setting up hanging drops the protein solution was filtered through a 0.2 µm filter. Aliquots of 1.5–2.0 µl of protein solution were mixed with the same volume of the crystallization reservoir solution to form each hanging drop. Typically, protein crystals formed at about 3–4 weeks and crystal growth was allowed to continue for 2–3 months.

X-ray Data Collection

A single crystal of PAF-AH was flash cooled directly into liquid nitrogen using a cryo-loop with no external cryo-protectant needed. Diffraction data of the native protein crystal were collected at the National Synchrotron Light Source (NSLS) beamline X29 using a Quantum 315 CCD detector from ADSC. The data collection was performed using a 3 sec per frame exposure, a 0.5° per frame oscillation, a total sweep width of 200° and a crystal to detector distance of 150.0 mm. The diffraction data were indexed and processed using the program HKL2000 [19].

RESULTS AND DISCUSSION

We have obtained crystals (Figure 1) of human plasma PAF-AH that diffract to high resolution. Statistics of X-ray diffraction data collected from this crystal to a resolution of 1.47 Å is shown in Table 1. The initial characterization of the C2 crystal form shown in Table 1 allowed a prediction that 2 subunits of PAF-AH are present in the asymmetric unit. The crystals have a predicted solvent content of 49.5% and a Matthews coefficient of 2.43 Å³Da⁻¹. A prediction of 2 subunits in the asymmetric unit is further corroborated by a self-rotation search of the C2 crystal form data (Figure 2) using the program POLARRFN [20].

The sampling of numerous crystallization conditions of plasma PAF-AH has ultimately led to crystals of high diffraction quality. The entire native data set, which is summarized in Table 1, was collected during approximately 30 min of exposure to the NSLS X29 beamline and showed minimal signs of X-ray decay. With reproducible crystals that allow us to consistently collect high resolution diffraction data, we have focused our efforts on obtaining phase information by MAD and SAD phasing techniques to solve the crystal structure of PAF-AH.

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ABBREVIATIONS

BOG	n-octyl-β-D-glucopyranoside
NSLS	National Synchrotron Light Source
PAF	platelet activating factor
PAF-AH	platelet activating factor acetylhydrolase
PLA2	phospholipase A2
Lp-PLA2	lipoprotein associated phospholipase A2

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Figure 1.
Crystal of human plasma PAF-AH that diffracted to a resolution of 1.47 Å.

Figure 2.

Self-rotation function plot at $\kappa = 180^\circ$. The rotation function was calculated with a radius of integration of 10 Å in the resolution range 20–5 Å using POLARRFN [20]. The two strongest peaks (relative height of 100, $\omega = 90.0$, $\phi = \pm 90.0$) indicate the crystallographic twofold symmetry, while the other two peaks (relative height of 87, $\omega = 61.9$, $\phi = 180$ and $\omega = 16.9$, $\phi = 0.0$) arise from the non-crystallographic twofold symmetry.

Table 1

X-ray data collection statistics for human plasma PAF-AH

	Native
Beamline	X29, NSLS, Brookhaven
Wavelength (Å)	1.1
Temperature (K)	100
Crystal dimension (μm)	150×100×50
Mosaicity (°)	0.47
Space group	C2
Unit cell (Å, °)	$a=116.18$, $b=83.06$, $c=96.71$, $\beta=115.09$
Resolution range (Å) ^a	50.0-1.47 [1.52-1.47]
Total Reflection	467,941
Unique reflection	131,413
Completeness	93.1 [53.8]
I/sigma	18.9 [1.6]
R_{merge}^b	0.058 [0.286]

^aValues in brackets are for the highest resolution shell.

^b $R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I_i(\text{hkl}) - \langle I_i(\text{hkl}) \rangle|}{\sum_{\text{hkl}} \sum_i I_i(\text{hkl})}$, where $I_i(\text{hkl})$ is ith intensity measurement of the reflection hkl, including symmetry related reflections, and $\langle I_i(\text{hkl}) \rangle$ is its average.