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Expression, purification and crystallization of human 5-lipoxygenase-activating protein with leukotriene-biosynthesis inhibitors

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The nuclear membrane protein 5-lipoxygenase-activating protein (FLAP) plays an essential role in leukotriene synthesis. Recombinant full-length human FLAP with a C-terminal hexahistidine tag has been expressed and purified from the cytoplasmic membrane of *Escherichia coli*. Diffraction-quality crystals of FLAP in complex with leukotriene-synthesis inhibitor MK-591 and with an iodinated analogue of MK-591 have been grown using the sitting-drop vapor-diffusion method. The crystals exhibit tetragonal symmetry ($P42_12$) and diffracted to a resolution limit of 4 Å.

1. Introduction

Leukotrienes are metabolites of arachidonic acid that are involved in respiratory and cardiovascular disease (Funk, 2001, 2005). Leukotriene synthesis is initiated by immune, allergic or inflammatory stimulation and the tightly regulated release of arachidonic acid from the nuclear membrane by cytosolic phospholipase A_2 (Murphy & Gijon, 2007). The 5-lipoxygenase-activating protein (FLAP) selectively transfers arachidonic acid to 5-lipoxygenase (5-LO) at the nuclear membrane (Dixon *et al.*, 1990; Miller *et al.*, 1990). Arachidonic acid is then converted to leukotriene A_4 by 5-LO in a two-step reaction that is enhanced by its interaction with FLAP (Radmark & Samuelsson, 2005; Miller *et al.*, 1990; Dixon *et al.*, 1990). Activation of 5-LO by FLAP is mediated by protein–protein interactions between these proteins (Plante *et al.*, 2006), suggesting that leukotriene-synthesis inhibitors, such as MK-591 (Young, 1999; Young *et al.*, 1993; Brideau *et al.*, 1992; Prasit *et al.*, 1993), compete with arachidonic acid for binding to FLAP (Mancini *et al.*, 1993).

FLAP is an 18 kDa integral nuclear membrane protein that belongs to the membrane-associated proteins of the eicosanoid and glutathione metabolism (MAPEG) superfamily (Bresell *et al.*, 2005). The human MAPEG superfamily also includes three microsomal glutathione transferases (mGST-1, mGST-2 and mGST-3), leukotriene C_4 synthase and microsomal prostaglandin E synthase (mPGES-1), all of which utilize glutathione for their enzymatic activity. In contrast, FLAP does not have any defined enzymatic activity and its ability to bind arachidonic acid and transfer it to 5-LO is not modulated by glutathione.

In this study, we report the expression, purification and crystallization of human FLAP in complex with the leukotriene-synthesis inhibitor MK-591 and of selenomethionyl-labeled FLAP in complex with compound A (an iodinated analogue of MK-591). This inhibitor was specifically designed to provide an additional source of experimental phase information to facilitate structure determination.

2. Materials and methods

2.1. Cloning

The gene encoding human FLAP (ALOX5AP) was PCR-amplified using the primers 5′-ATACCATGGGCATGGATCAAGAAACTGTAGGC-3′ and 5′-CGGGATCCTTAATGATGATGATGATGATGATGGGGAATGAGAAGTAGAGGG-3′, introducing a non-

cleavable C-terminal hexahistidine tag following the full-length FLAP protein sequence. An alternative construct with a thrombin-cleavage site before the C-terminal hexahistidine tag was amplified using the primers 5′-ATACCATGGGCATGGATCAAGAAACTGTAGGC-3′ and 5′-CGGGATCCTTAATGATGATGATGATGATGGCTGCCGCGCGGCACCAGGGGAATGAGAAGTAGAGGGG-3′. The resulting PCR products were digested with *Nco*I and *Bam*HI (New England Biolabs) and ligated into the bacterial expression vector pET28a (Novagen), producing $FLAP_{1–161}$6×His/pET28a and $FLAP_{1–161}$thrombin/6×His/pET28a.

A baculovirus expression construct for full-length human FLAP was generated by introducing a linker into the *Bam*HI site of expression vector pBAC.1 (Novagen) using the primers 5′-GATCGCGCCACCATGCACCACCACCACCACCACCTGGTGCCACGCGGTTCTCATATGGAAATTCCG-3′ and 5′-GATCCGGAATTTCCATATGAGAACCGCGTGGCACCAGGTGGTGGTGGTGGTGGTGCATGGTGGCGC-3′. This linker removes the 5′ *Bam*HI site of vector pBAC.1, creates a Kozak sequence and introduces a starting methionine codon before the N-terminal 6×His tag, followed by a thrombin-cleavage site and an *Nde*I restriction site. The FLAP gene sequence was PCR-amplified using the primers 5′-CATATGGATCAAGAAACTGTAGGC-3′ and 5′-GAATTCTTAGGGAATGAGAAGTAGAG-3′. The resulting product was digested with *Nde*I and *Eco*RI (New England Biolabs) and ligated into the modified pBAC.1 vector, producing $FLAP_{1–161}$thrombin/6×His/pBAC.1.

2.2. Insect-cell expression of FLAP

*Sf*9 cells were transfected with $FLAP_{1–161}$thrombin/6×His/pBAC.1 following the manufacturer's recommended protocols (BD Biosciences). 2 µg recombinant pBAC.1 plasmid and 0.5 µg linearized BaculoGold virus DNA were added to a T25 flask containing $\sim3 \times 10^6$ ml^{-1} *Sf*9 cells in 5 ml serum-supplemented medium for one week at 300 K. A low multiplicity of infection viral stock ($\sim1 \times 10^8$ pfu ml^{-1}) was prepared by infecting 400 ml of *Sf*9 cells at $\sim1.2 \times 10^6$ ml^{-1} with 0.5 ml primary virus and growth was continued until the majority of cells were no longer viable (typically 7–10 d). Baculovirus-infected *Sf*9 cells were used for overexpression and were grown at 300 K. Samples were generated by infecting 400 ml *Sf*9 cells at $\sim1 \times 10^6$ ml^{-1} with 8 ml primary virus. Cells were harvested 4–5 d after infection by centrifugation at 2500*g* for 10 min and were frozen at 253 K.

2.3. Bacterial expression of FLAP

Bacterial expression vectors were transformed into *Escherichia coli* BL21(DE3) (Novagen). Cells were grown in medium supplemented with 50 mg l^{-1} kanamycin at 310 K until the OD_{600} reached ∼1.0. Protein expression was induced by the addition of 1.5 m*M* isopropyl β-D-1-thiogalactopyranoside for 2 h at 303 K. Bacterial cells were harvested by centrifugation, washed once with phosphate-buffered saline pH 7.0 and stored at 193 K. Selenomethionyl-labeled protein was obtained by expressing FLAP in *E. coli* B834(DE3) cells (methionine auxotroph) grown in M9 minimal media supplemented with 40 mg l^{-1} selenomethionine (Doublié, 1997) and the selenomethionyl-labeled protein was prepared in the same way as the native protein.

2.4. Purification of recombinant FLAP

Bacterial or insect-cell pellets were thawed, resuspended in lysis buffer [20 m*M* Tris–HCl pH 7.4, 50 m*M* NaCl, 10%(*v*/*v*) glycerol, 1 m*M* Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and EDTA-free protease-inhibitor tablets (Roche)] and lysed using an EmulsiFlex-C5 system (Avestin). Crude cell lysate was cleared by centrifugation at 7000 rev min^{-1} for 30 min at 277 K. Membranes were purified by centrifugation at 35 000*g* for 2.5 h at 277 K and resuspended in lysis buffer. Recombinant FLAP was solubilized with 2%(*w*/*v*) dodecyl β-D-maltopyranoside (DDM; Anatrace) for 45 min at room temperature with gentle agitation and centrifuged at 40 000*g* for 30 min at 277 K. Supernatant containing 6×His-tagged FLAP was mixed with 2 ml Ni–NTA agarose (Qiagen) for 1 h at 277 K. The slurry was loaded by gravity into an empty column and washed with 20 m*M* Tris–HCl pH 7.4, 150 m*M* NaCl, 20 m*M* imidazole, 1 m*M* TCEP, 10%(*v*/*v*) glycerol and 0.1%(*w*/*v*) DDM. Detergent exchange was performed by extensively washing the Ni–NTA agarose with 20 m*M* Tris–HCl pH 7.4, 150 m*M* NaCl, 1 m*M* TCEP, 10%(*v*/*v*) glycerol and 0.25%(*v*/*v*) $C_{12}E_8$ prior to elution with 20 m*M* Tris–HCl pH 7.4, 150 m*M* NaCl, 250 m*M* imidazole, 1 m*M* TCEP, 10%(*v*/*v*) glycerol and 0.25%(*v*/*v*) $C_{12}E_8$. FLAP was further purified by gel-filtration chromatography using a Superdex 200 column (GE Healthcare) equilibrated with 5 m*M* Tris–HCl pH 7.4, 20 m*M* NaCl, 1 m*M* TCEP, 2%(*v*/*v*) glycerol and 0.1%(*v*/*v*) $C_{12}E_8$. Protein-containing fractions were pooled and concentrated using a centrifugal filter device (Millipore, 10 kDa molecular-weight cutoff). Protein homogeneity and identity were confirmed by SDS–PAGE, Western blotting, mass spectrometry and N-terminal sequencing. A previously

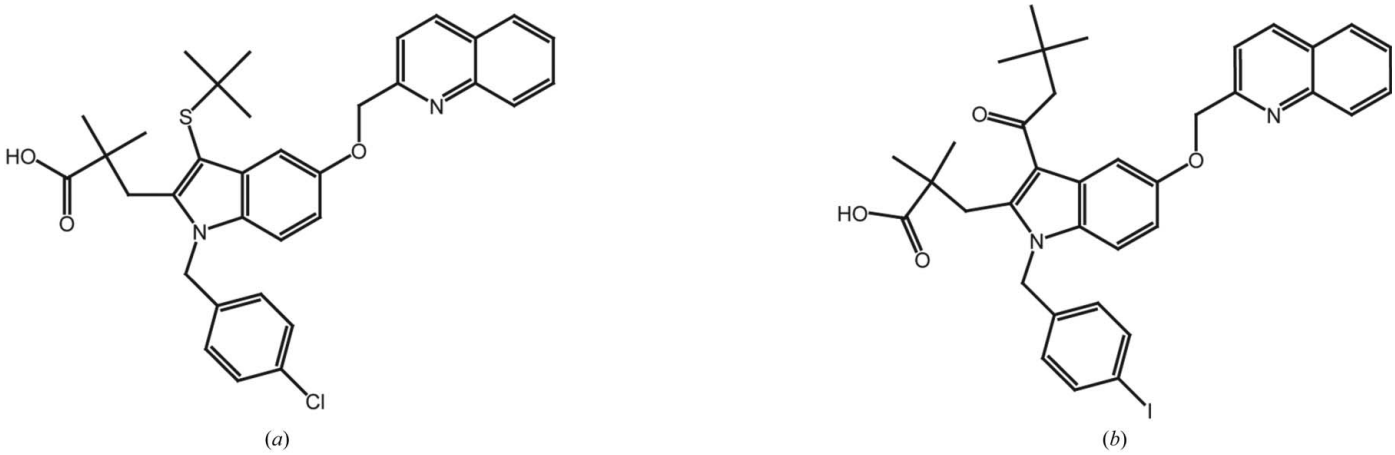

Figure 1
Chemical structures of (*a*) MK-591 and (*b*) compound A.

described competitive radio-ligand-binding assay was used to confirm the leukotriene-synthesis inhibitor-binding profile of detergent-solubilized FLAP and purified membranes containing membrane-embedded FLAP (Vickers *et al.*, 1992; Charleson *et al.*, 1992).

We evaluated various growth media and the effect on the yield of purified protein. We recovered ~1.1 mg l^{-1} using M9 minimal medium, ~1.7 mg l^{-1} using LB medium, ~2 mg l^{-1} using LB medium supplemented with 1× phosphate-buffered saline pH 7.0 and ~4 mg l^{-1} using LB medium supplemented with 1× phosphate-buffered saline pH 7.0, 10 g l^{-1} glycine and 2%(*v*/*v*) glycerol. It should also be noted that various bacterial expression constructs with N-terminal 6×His tags grown under similar conditions expressed very little, if any, protein.

2.5. Crystallization

Concentrated protein solution was equilibrated with leukotriene-biosynthesis inhibitor MK-591 or compound A (1:1.1 protein: inhibitor ratio) for 4 h at 277 K (Fig. 1). We attempted to remove the C-terminal 6×His tag using thrombin; however, the incubation of purified $FLAP_{1–161}$thrombin/6×His protein unexpectedly produced several lower molecular-weight cleavage products. Sparse-matrix screening using commercial screens was initially carried out with $FLAP_{1–161}$thrombin/6×His protein in a variety of primary detergents. Preliminary crystals appeared under several conditions using the hanging-drop vapor-diffusion method at 293 K. However, all initial crystals diffracted poorly. Additional sparse-matrix screening was subsequently performed with $FLAP_{1–161}$6×His protein. Although both recombinant proteins crystallized under similar conditions, the diffraction properties of $FLAP_{1–161}$6×His crystals were superior to those of $FLAP_{1–161}$thrombin/6×His crystals.

We evaluated whether the source of recombinant protein (insect *versus* bacterial cells) and bound endogenous lipids might affect the internal crystalline order of $FLAP_{1–161}$6×His crystals. The absence or presence of endogenous lipids in purified integral membrane-protein samples has a well documented effect on membrane-protein crystallization (Seddon *et al.*, 2004; Palsdottir & Hunte, 2004; Lee, 2004). Mass-spectrometric analysis of purified $FLAP_{1–161}$6×His protein expressed in insect cells revealed the presence of bound phosphoinositides. In contrast, no bound lipids were observed with $FLAP_{1–161}$6×His protein expressed in *E. coli*. PIP strips (nitrocellulose membranes to which a selection of lipids have been noncovalently immobilized; Echelon Biosciences) were used to define which phosphoinositides bound to FLAP. Although individual and combinations of these phospholipids were added to $FLAP_{1–161}$6×His protein expressed in *E. coli* prior to crystallization, these experiments failed to improve the diffraction properties of these crystals. Moreover, we were unable to crystallize $FLAP_{1–161}$6×His purified from insect-cell membranes.

Figure 2
Crystal of (K148A)$FLAP_{1–161}$6×His in complex with MK-591. Average crystal dimensions were approximately 400 × 200 × 200 µm.

We next utilized surface-entropy reduction engineering in an attempt to obtain crystals with improved diffraction properties (Derewenda, 2004; Derewenda & Vekilov, 2006). Two lysine residues (Lys116 and Lys148) and one aspartate residue (Asp62) were mutated to alanine and both cysteine residues (Cys60 and Cys78) found in FLAP were jointly mutated to either alanine or serine, generating (K116A)$FLAP_{1–161}$6×His, (K148A)$FLAP_{1–161}$6×His, (D62A)$FLAP_{1–161}$6×His, (C60A/C78A)$FLAP_{1–161}$6×His and (C60S/C78S)$FLAP_{1–161}$6×His. Given the apparent importance of the uncleaved C-terminal His tag for the crystallization of $FLAP_{1–161}$-6×His, we also designed two C-terminal deletion FLAP mutants (Δ148–161 and Δ154–161) in the (C60A/C78A)$FLAP_{1–161}$6×His background, producing (C60A/C78A)$FLAP_{1–147}$6×His and (C60A/C78A)$FLAP_{1–153}$6×His. Crystallization trials with this series of recombinant proteins revealed substantial differences in crystal morphology, size, reproducibility and diffraction properties. No crystals were obtained with either (C60A/C78A)$FLAP_{1–147}$6×His or (C60A/C78A)$FLAP_{1–153}$6×His.

Our best results were obtained by mixing equal volumes (1 + 1 µl) of (K148A)$FLAP_{1–161}$6×His protein at 8 mg l^{-1} with a reservoir solution containing 0.1 *M* sodium citrate pH 5.6, 0.32 *M* lithium chloride, 6%(*w*/*v*) PEG 6000, 1 m*M* TCEP, 0.25%(*v*/*v*) C_8E_4 and 0.1%(*v*/*v*) $C_{12}E_8$ at 293 K. It should be noted that (K148A)-$FLAP_{1–161}$6×His protein purified in one step (immobilized metal-chelate affinity chromatography) generated crystals with superior diffraction properties compared with protein that had been purified in two steps (immobilized metal-chelate affinity and gel-filtration chromatography). Size-exclusion chromatography is likely to remove the endogenous lipids that are needed to form superior-quality protein crystals. Optimization of crystal size, appearance, mechanical stability and reproducibility were conducted using sitting-drop vapor-diffusion crystallization plates. Small crystals appeared overnight and grew to maximum dimensions of 400 × 200 × 200 µm within two weeks. Selenomethionyl-labeled (K148A)$FLAP_{1–161}$6×His crystals behaved similarly to the native protein.

2.6. Data collection and processing

Prior to data collection, the concentration of glycerol in the reservoir solution was slowly raised to ~20%(*v*/*v*) over 2 h at 277 K, after which crystals were mounted in cryoloops and vitrified by direct immersion into liquid nitrogen. There was considerable variation in crystal quality and extensive in-house screening of crystals was necessary to find candidates suitable for synchrotron data collection. All X-ray diffraction data were collected at 100 K using a cryostream apparatus with synchrotron radiation at IMCA-CAT beamline 17-ID, Advanced Photon Source and at beamline X25, National Synchrotron Light Source. Diffraction data were collected from native (K148A)$FLAP_{1–161}$6×His crystals in complex with MK-591 at 1.0000 Å at IMCA-CAT beamline 17-ID. Single-wavelength anomalous diffraction data were collected from a selenomethionyl-labeled (K148A)$FLAP_{1–161}$6×His crystal in complex with compound A at the peak of the selenium X-ray fluorescence spectrum (0.9797 Å) at NSLS beamline X25. At this wavelength iodine has a limited anomalous signal and has a theoretical anomalous scattering values of $f' \simeq 0$ and $f'' \simeq 3$. 120° of data were collected using 0.5° oscillations

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Compound A	MK-591
Beamline	NSLS X25	APS 17-ID
Detector	ADSC Quantum 315	ADSC Quantum 210
Temperature (K)	100	100
Resolution (Å)	50–4.0 (4.24–4.0)	50–4.25 (4.51–4.25)
Wavelength (Å)	0.9797	1.0000
Total No. of reflections	312309	161633
Unique reflections	20007	16785
Completeness (%)	99.3 (99.9)	99.1 (99.6)
Redundancy	15.5	9.6
Average $I/\sigma(I)$	20.6 (3.29)	22.7 (4.57)
Space group	$P42_12$	$P42_12$
Unit-cell parameters (Å)	$a = b = 180.66$, $c = 139.99$	$a = b = 180.60$, $c = 140.57$
$R_{\rm merge}$ (%)	11.1 (69.9)	5.8 (55.5)

for the native and anomalous diffraction data sets, respectively. All diffraction data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Discussion

Full-length human FLAP with a C-terminal hexahistidine tag has been expressed in *E. coli* and the recombinant protein has been purified to homogeneity. Diffraction-quality crystals were grown using the sitting-drop vapor-diffusion method. Surface-entropy reduction engineering was required to obtain crystals of FLAP with acceptable diffraction properties. Introducing a single point mutation at position 148 of the FLAP protein sequence (K148A) produced crystals of sufficient size and mechanical stability to begin the structure-determination process. Native (K148A)FLAP$_{1-161}$6×His crystals in complex with leukotriene-synthesis inhibitor MK-591 (Fig. 2) diffract to 4.25 Å and belong to the tetragonal space group $P42_12$, with unit-cell parameters $a = b = 180.60$, $c = 140.57$ Å. Selenomethionyl-labeled crystals of (K148)FLAP$_{1-161}$6×His in complex with compound A diffract to 4 Å and belong to the tetragonal space group $P42_12$, with unit-cell parameters $a = b = 180.66$, $c = 139.99$ Å. There are six molecules per asymmetric unit with a $V_{\rm M}$ of 4.97 Å^3 Da^{-1}, corresponding to a solvent content of approximately 75%. Data-collection statistics are summarized in Table 1.

We specifically utilized an iodinated analogue of leukotriene-synthesis inhibitor MK-591, compound A, to provide an additional source of experimental phase information. Cocrystallization of selenomethionyl-labeled (K148A)FLAP$_{1-161}$6×His with this inhibitor was essential to correctly interpret the experimental electron-density map, assign the FLAP protein sequence, build the model, define the location of the leukotriene inhibitor-binding site and to establish the inhibitor-binding stoichiometry of FLAP. Details of the solution of the selenium substructure, macromolecular refinement and the structure of (K148A)FLAP$_{1-161}$6×His in complex with MK-591 and compound A are described elsewhere (Ferguson *et al.*, 2007).

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