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Improving protein crystal quality by selective removal of a Ca^{2+}-dependent membrane-insertion loop

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Lipoxygenases (LOXs) catalyze the regiospecific and stereospecific dioxygenation of polyunsaturated membrane-embedded fatty acids. A Ca^{2+}-dependent membrane-binding function was localized to the amino-terminal C2-like domain of 8*R*-lipoxygenase (8*R*-LOX) from the soft coral *Plexaura homomalla*. The 3.2 Å crystal structure of 8*R*-LOX and spectroscopic data suggested that Ca^{2+} stabilizes two membrane-insertion loops. Analysis of the protein packing contacts in the crystal lattice indicated that the conformation of one of the two loops complicated efforts to improve the resolution of the X-ray data. A deletion mutant of 8*R*-LOX in which the corresponding membrane-insertion loop is absent (Δ41–45:GSLOX) was engineered. Removal of the membrane-insertion loop dramatically increases the protein yield from bacterial cultures and the quality of the crystals obtained, resulting in a better than 1 Å improvement in the resolution of the diffraction data.

1. Introduction

Owing to their pivotal roles in the biosynthesis of leukotrienes and other biologically active eicosanoids, lipoxygenases (LOXs; Kuhn *et al.*, 2005; Brash, 1999) are targets for the development of specific inhibitors to modulate the physiological and pathological effects of these potent signalling compounds (Werz & Steinhilber, 2006; Dahlen, 2006). The strict regiospecificity (reviewed in Schneider *et al.*, 2007) of the various lipoxygenases indicates that selective targeting of a single LOX isozyme is feasible, a requisite feature of an effective therapeutic strategy. The human isozyme 5-lipoxygenase (5-LOX) catalyzes the first step in the synthesis of leukotriene bronchoconstrictors and as such is a target for drugs to treat asthma. Lipoxygenases are iron enzymes and thus can be inhibited by iron chelators or redox-active compounds. However, the redox mode of inhibition is highly nonspecific and consequently replete with undesirable side effects. In order to engineer specificity into novel inhibitors, structural information that provides insight into substrate recognition is required.

To date, the structures of the 15-lipoxygenase from rabbit reticulocytes (Gillmor *et al.*, 1997), an 8*R*-lipoxygenase from the soft coral *Plexaura homomalla* (Oldham *et al.*, 2005) and four plant enzymes (Boyington *et al.*, 1997; Minor *et al.*, 1996; Skrzypczak-Jankun *et al.*, 1997; Youn *et al.*, 2006) have been published. The plant lipoxygenases have afforded a robust model system to understand mechanistic aspects of this class of iron enzymes in the context of high-resolution crystal structures. In addition, structures of the plant enzymes have been determined in the absence and presence of a substrate analog. However, there are fundamental differences in the substrate-binding mode for the plant and animal enzymes, as the counterparts of the amino acids that influence regiospecificity in 15-LOX are not positioned to do so in the plant enzymes (Skrzypczak-Jankun *et al.*, 2001). Furthermore, the plant enzymes are ∼200 amino acids larger than the animal enzymes and much of the additional polypeptide is located near the putative entrance to the catalytic site. Additional structures of animal lipoxygenases are

required in order to understand substrate acquisition and reaction specificity.

Currently, there is no structure of an animal lipoxygenase with substrate that can illuminate the mechanisms of regiospecificity and stereospecificity or at least provide a structural framework for the design of experiments to determine such details. An obvious choice for these studies would be human 5-LOX because of its role in the synthesis of leukotrienes. However, human 5-LOX is notoriously unstable, losing 50% of its activity within 10 h unless glutathione reductase and superoxide dismutase are also present (Hammarberg *et al.*, 2001; Zhang *et al.*, 1994). The coral 8*R*-LOX, on the other hand, retains >95% of its activity after storage at 277 K for four months. Furthermore, the coral enzyme has ∼41% sequence identity to human 5-LOX. Importantly, no other human LOX isozyme is more than 42% identical to 5-LOX. Furthermore, like 5-LOX, 8*R*-LOX is targeted to membranes by Ca^{2+} (Chen & Funk, 2001; Kulkarni *et al.*, 2002; Oldham *et al.*, 2005). The coral enzyme can provide a tractable system with which to address the lack of a model for enzyme–substrate recognition in animal lipoxygenases if a high-resolution structure can be obtained.

The previously reported 3.2 Å structure of 8*R*-LOX was determined using crystals grown in the presence of 100 m*M* Ca^{2+} (PDB code 2fnq; Oldham *et al.*, 2005). Although this resolution is modest, the quality of the data, as judged from isotropic diffraction and low mosaicity, suggested that crystals that diffract to higher resolution could be obtained. However, to date refinement of the crystallization conditions has not resulted in improved crystal quality. Inspection of the packing in the 8*R*-LOX crystals revealed that a putative membrane-insertion loop might have been frustrating attempts to obtain crystals which diffract to high resolution: bound Ca^{2+} appears to stabilize this loop, which emanates from the C2-like domain. The loop includes Trp41 and Phe42, which are wedged in a hydrophobic cavity on the surface of a symmetry mate. This protein–protein interaction appears to hold the symmetry mates at 'arm's length' and precludes additional contact points between neighboring molecules that might involve stable elements of secondary structure. Furthermore, at the high concentration of protein necessary for crystallization it is likely that this loop–protein interaction will dominate protein–protein interactions even in the absence of Ca^{2+}. The inherent flexibility of an unstabilized loop would further limit crystal quality, so elimination of Ca^{2+} from the crystallization conditions is not likely to be an option. Therefore, a modified form of the enzyme was constructed in which Trp41 and Phe42 were deleted. As described below, the mutant enzyme was soluble, enzymatically active in a membrane-free assay and yielded crystals that diffracted to 2.0 Å.

2. Materials and methods

2.1. Molecular biology

A pET-3a plasmid expressing 8*R*-LOX under control of the T7 promoter (Oldham *et al.*, 2005) was used as the template for mutagenesis. The entire plasmid was amplified by whole-plasmid polymerase chain reaction. Primers were designed to delete the sequence between Lys40 and Phe46. Codons for glycine and serine were added to the 5′ ends of the forward and reverse primers, respectively. These codons provided a restriction site that could be used to distinguish between wild-type and mutant plasmids. Furthermore, Gly and Ser are commonly found in reverse turns and should be readily accommodated in the shortened loop. Consequently, the amino-acid sequence of the mutant protein between amino acids 40 and 46 was changed from KWFHNDF to KGSF. Although coral 8*R*-LOX occurs naturally as the C-terminal 683 amino acids of a 1066-amino-acid bifunctional protein (Koljak *et al.*, 1997), for ease of comparison to animal lipoxygenases the residue-numbering convention used here refers only to the LOX domain as in Oldham *et al.* (2005). Hence, the mutant enzyme was designated Δ41–45:GSLOX.

Table 1
Data-collection statistics of Δ41–45:GSLOX.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 104.0, b = 170.2, c = 104.5, β = 95.9
Monomers per ASU	4
Matthews coefficient (Å^3 Da^{-1})	2.9
Solvent content (%)	57
Diffraction limit (Å)	2.0
Resolution (Å)	30–2.00 (2.07–2.00)
Unique reflections	243328 (3259)
$R_{\rm merge}$†	0.058 (0.274)
Completeness (%)	99.0 (98.6)
Redundancy	4.0 (4.1)
$\langle I/\sigma(I)\rangle$	23.6 (5.7)

† $R_{\rm merge} = \sum_h \sum_i |I_i - \langle I\rangle| / \sum_h \sum_i I_i$, where I_i is the intensity of the ith observation of reflection h and $\langle I\rangle$ is the mean intensity of the reflection from multiple observations.

2.2. Protein expression and purification

The Δ41–45:GSLOX construct in pET-3a was transformed into *Escherichia coli* BL21(DE3) cells (Invitrogen). Colonies were picked and grown overnight in 20 ml Luria broth at 310 K and then transferred into 500 ml of the auto-inducing medium ZYM-5052 (Studier, 2005). Cultures were grown at 293 K until the absorbance at 600 nm was stable for 4 h. Cells were harvested by centrifugation and frozen at 193 K. Cell pellets were resuspended in Bugbuster (Novagen) along with DNase I and the protease inhibitors leupeptin and pepstatin. The suspension was then sonicated and centrifuged. The supernatant was applied onto Ni-bound chelating agarose (GE Healthcare) and the column was washed extensively with binding buffer (20 m*M* Tris pH 8, 500 m*M* NaCl). The protein was eluted with an imidazole gradient (0–200 m*M* in binding buffer). Fractions were checked for purity by SDS–PAGE and analyzed for activity as described below. Further purification on a Mono-Q anion-exchange (GE Healthcare) resin using a gradient of 0–500 m*M* NaCl in 20 m*M* Tris pH 8, along with size-exclusion chromatography on Superdex-200 (GE Healthcare) in 20 m*M* Tris pH 8, 150 m*M* NaCl, yielded purified enzyme. Yields of 40 mg highly purified Δ41–45:GSLOX per litre of culture were routinely obtained.

The enzymatic activity of Δ41–45:GSLOX in the absence and presence of liposomes was monitored by following the absorbance of product at 235 nm in an assay previously described by Oldham *et al.* (2005). Measurements were performed in an Applied Photophysics Ltd model SX17MV stopped-flow spectrometer.

2.3. Crystallization and data collection

Crystal screens based on the method of Jancarik & Kim (1991) were performed to identify conditions that favored crystal growth. Crystals of Δ41–45:GSLOX were obtained by vapor diffusion from hanging drops (4 µl) containing a 1:1(*v*:*v*) ratio of protein solution to reservoir solution. The reservoir solution contained 6–8%(*w*/*v*) polyethylene glycol 8000, 5%(*w*/*v*) glycerol, 1%(*w*/*v*) Tween 20 and 200 m*M* $CaCl_2$ in 100 m*M* imidazole acetate pH 8.0. Crystals formed in approximately 24 h at 293 K in space group $P2_1$ (unit-cell parameters a = 104.0, b = 170.2, c = 104.5 Å, β = 95.9°). With four monomers per asymmetric unit, the solvent content was 57.8% and the Matthews coefficient was 2.9 Å^3 Da^{-1} (Matthews, 1968). The crystals were transferred to a cryobuffer consisting of 10% poly-

ethylene glycol 8000, 30% glycerol, 1% Tween 20 and 20 m*M* $CaCl_2$ in 100 m*M* imidazole acetate pH 8.0 and were immediately mounted and flash-cooled in a stream of cold nitrogen gas (100 K). X-ray diffraction data of Δ41–45:GSLOX were collected using a MAR165 CCD detector (MAR USA) on the Gulf Coast Protein Crystallography Consortium-PX beamline at the Center for Advanced Microstructures and Devices (CAMD, Baton Rouge, LA, USA). Data-collection statistics are shown in Table 1.

3. Results and discussion

The membrane-binding properties of some animal lipoxygenases can complicate efforts to obtain crystals that diffract to high resolution. In the previously reported 8*R*-LOX structure (Oldham *et al.*, 2005), a putative Ca^{2+}-stabilized hydrophobic membrane-insertion loop is involved in a packing contact that appears to preclude lattice-stabilizing protein–protein interactions (Fig. 1). The membrane-insertion loop in question, which is common to the 8*R*- and 5-lipoxygenases, is not present in rabbit 15-lipoxygenase, for which a 2.4 Å resolution structure has been determined (Gillmor *et al.*, 1997). Its absence in 15-LOX is an indication that the loop sequence is not required for the structural integrity of the enzyme. The extended structure of the membrane-insertion loop, combined with the fact that an equivalent loop is not present in rabbit reticulocyte 15-LOX (Fig. 2), led to the design and construction of an 8*R*-LOX mutant in which the loop was absent. Because Δ41–45:GSLOX was soluble, a yield of 40 mg purified protein per litre of culture was obtained. In contrast, the bulk of the wild-type protein was in the insoluble fraction and the yield of purified wild-type enzyme was only ∼8 mg per litre of culture. The deletion-loop enzyme had 110% of the wild-type activity in a liposome-free assay. In contrast, it displayed only 15% of the activity of the native enzyme when assayed in the presence of liposomes and Ca^{2+}, conditions under which the enzyme must bind membranes to access substrate. These results suggest that the loop deletion disrupts the membrane-binding activity of the enzyme as expected, but does not diminish its enzymatic activity.

A search of crystallization conditions with commercial screens yielded conditions for optimization. Refinement of these initial conditions, which were remarkably similar to those that produced the crystals of wild-type enzyme (Oldham *et al.*, 2005), yielded crystals belonging to space group $P2_1$ that diffracted to 2.0 Å with a signal-to-noise ratio of 5.7 in the highest shell. The wild-type crystals diffracted to only 3.2 Å at the same X-ray source (CAMD), with a signal-to-noise ratio that was no better than that reported for the rotating-anode data (2.3 in the highest shell) of Oldham *et al.* (2005). Removal of the extended membrane-binding loop resulted in greater than a 1.0 Å improvement in the resolution of the diffraction data.

The effects of the substitution of surface amino acids to improve crystals of soluble proteins have previously been evaluated (D'Arcy *et al.*, 1999; Charron *et al.*, 2002). In addition, protein engineering has been applied to membrane-associated proteins for the purpose of solubilization and crystallization. For example, the solubility of eukaryotic cytochrome P450s was improved by the deletion of the amino-terminal membrane-insertion helix (Scott *et al.*, 2001) as well as the mutation of an internal loop that plays a role in membrane association (Cosme & Johnson, 2000; Hsu & Wang, 2003). However, the length of the membrane-binding loop remained unchanged in the engineered protein and in at least one case the enzyme activity was compromised (Hsu & Wang, 2003).

The results presented here indicate that removal of a membrane-insertion loop required for Ca^{2+}-dependent membrane targeting of 8*R*-LOX improves the solubility of the protein, while the enzyme activity in a membrane-free assay is unaffected (data not shown). More importantly, the diffraction of crystals of Δ41–45:GSLOX is significantly improved over that of wild-type crystals. While the diffraction limit for the wild-type protein crystals was 3.2 Å, the Δ41–45:GSLOX crystals obtained under similar conditions diffract to 2.0 Å. Thus, removal of membrane-insertion loops, which can often be predicted through multiple sequence alignment, should be a useful

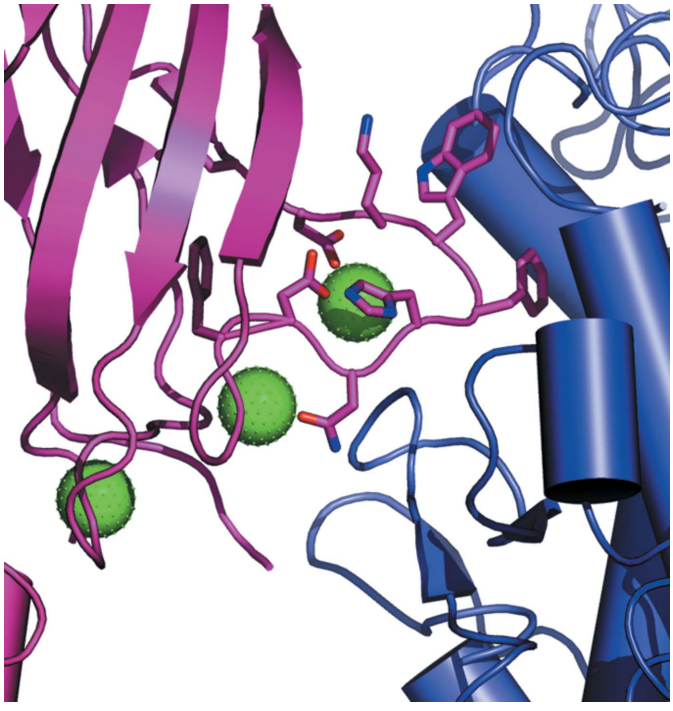

Figure 1
Calcium ions (green) stabilize the membrane-binding loop that contains Trp41–Phe42. Trp41 and Phe42 mediate a packing contact between symmetry mates (pink and blue) in the crystal lattice (PDB code 2fnq).

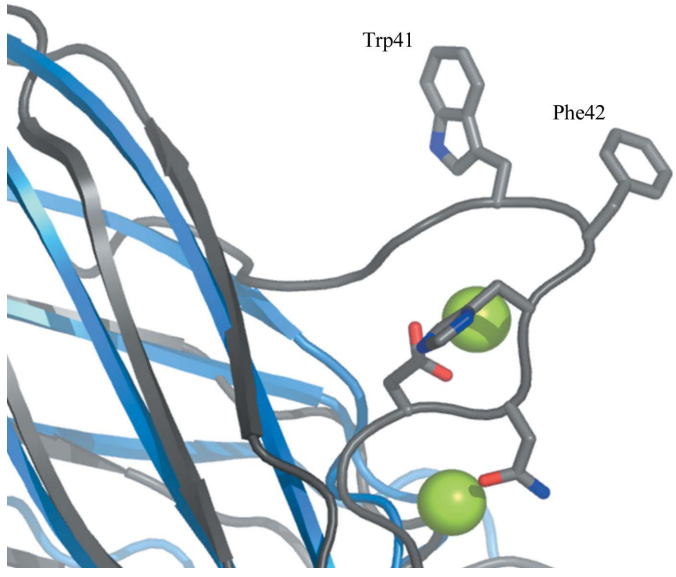

Figure 2
Superposition of 8*R*-LOX (gray) on 15-LOX (PDB code 1lox; blue). The 8*R*-LOX Ca^{2+}-stabilized membrane-insertion loop containing the WF sequence is absent in 15-LOX (Ca^{2+}, green).

strategy for increasing the probability of obtaining high-quality crystals of Ca^{2+}-dependent membrane-binding proteins.

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