Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, expression, purification, crystallization and preliminary X-ray diffraction studies of a 12R-LOX–chaperone complex

Gouri Deb,* Karen Boeshanes, William K. Idler and Bijan Ahvazi

X-ray Crystallography Facility, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institute of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

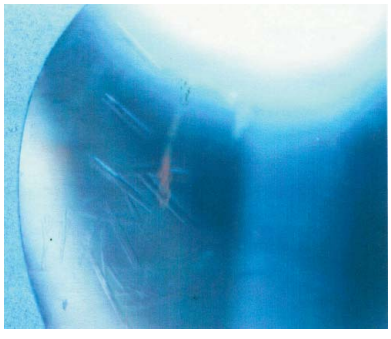
Correspondence e-mail: gou5skm2001@yahoo.com

Received 24 March 2011
Accepted 2 June 2011

Lipoxygenases are a family of nonheme iron-containing dioxygenases. An *Escherichia coli* expression system producing the bacterial chaperones GroES and GroEL was engineered and successfully used to produce large quantities of recombinant human 12R-LOX (LOXR; MW 80.34 kDa; 701 amino-acid residues). The co-overproduction of the two chaperones with 12R-LOX resulted in increased solubility of 12R-LOX and allowed the purification of milligram amounts of active enzyme for structural studies by X-ray diffraction. The lipoxygenase protein was purified on an affinity column and a gel-filtration column with chaperone protein (MW 57.16 kDa). The LOXR–chaperone complex was crystallized with ligand by the hanging-drop vapor-diffusion method using 1.5 *M* ammonium hydrogen phosphate as precipitant. The crystals belonged to the monoclinic system, space group $P2_1$, with unit-cell parameters a = 138.97, b = 266.11, c = 152.26 Å, β = 101.07°. Based on the calculated Matthews coefficient (3.1 Å^3 Da^{-1}), it is estimated that one molecule of LOXR complexed with two molecules of chaperone is present in the asymmetric unit of the crystal lattice. X-ray diffraction data were collected to 4 Å resolution using synchrotron radiation.

1. Introduction

Lipoxygenases are a class of nonheme iron-containing dioxygenases that catalyze the conversion of arachidonic acid and other polyunsaturated fatty acids to their hydroperoxy derivatives (Brash, 1999; Kühn & Borngräber, 1999). They possess regiospecificity in their interaction with substrates and on this basis have been designated as arachidonate 5-, 8-, 12- and 15-lipoxygenases (5-LOX, 8-LOX, 12-LOX and 15-LOX, respectively). The four distinct enzymes insert oxygen at carbon 5, 8, 12 or 15 of arachidonic acid to produce 5-, 8-, 12- or 15-hydroperoxyeicosatetraenoic acid (HPETE), respectively (Cao & Prescott, 2002; Chandrasekharan *et al.*, 2002; Kühn & Thiele, 1999). Most research has focused on the regulation and biological relevance of the LOX isoforms rather than on the structural biology of the enzymes. Although the primary structures of more than 20 mammalian LOX isoforms have been reported, only the structure of rabbit 15-LOX has been determined (Kühn *et al.*, 2005; Gillmor *et al.*, 1997). No LOX structures with bound substrate are available. Additional crystal structures of LOX would increase knowledge of the structural basis of the LOX reaction. Of the six lipoxygenases in the human genome, 15-LOX-2, 12R-LOX and e-LOX-3 show preferential expression in epithelial tissues, where it has been suggested that they play a role in regulation or modulation of normal proliferation and differentiation of epithelial cells and keratinocytes (Krieg *et al.*, 2001). 12R-LOX is expressed exclusively in skin. Synthesis of its product, 12R-HETE, is up-regulated in psoriasis (Hammarström *et al.*, 1975; Camp *et al.*, 1983; Woollard, 1986; Baer & Green, 1993). Mutations in the coding sequences of the e-LOX-3 and 12R-LOX genes have also been linked to development of the inherited skin disease non-bullous congenital ichthyosiform erythroderma (NCIE; Jobard *et al.*, 2002). *Escherichia coli* is commonly used as a host for protein expression owing to its convenience and simplicity. However, the overexpression of foreign proteins often results in the formation of inclusion bodies owing to improper folding of the



expressed protein. In some cases, the co-expression of molecular chaperones assists in protein folding and increases the production of active proteins (Lee *et al.*, 2004; Mizobata *et al.*, 2000; Nishihara *et al.*, 1998, 1999; Sareen *et al.*, 2001; Schlieker *et al.*, 2002). Here, we present the soluble expression of active 12R-LOX with a molecular chaperone and its purification, crystallization and preliminary X-ray diffraction analysis in order to gain knowledge of the structural basis of the interaction of 12R-LOX with the ligand.

2. Materials and methods

2.1. Enzymes and plasmids

Competent *E. coli* BL21 (DE3), the PCR cloning kit, PCR primers, *Nde*I and *Xho*I restriction enzymes and T4 polynucleotide kinase were obtained from Invitrogen. The pET15b vector was obtained from Novagen. Carbenicillin, ampicillin and chloramphenicol, isopropyl β-D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and tetracycline were obtained from Sigma. The LB medium and the bacterial growth-medium products were obtained from Qbiogene. HTP-Biogel resin was obtained from Biotech. The Hi-Prep 16/10 and Mono Q HR columns were obtained from Amersham Pharmacia Biotech.

2.2. Construction of the pET15b expression vector

The full open reading frame comprising the human 12R-LOX gene was PCR-amplified from the pQE80 plasmid using the forward primer CTCTCTCATATCGAA containing a 3′ *Nde*I site and the reverse primer TCTCTCTCTCTCGAG containing a 5′ *Xho*I site. The PCR-amplified product was digested with restriction enzymes (*Nde*I and *Xho*I) and the digestion product was purified and then added into similarly digested pET15b vector. The PCR-amplified 12R-LOX DNA was isolated from a 1.0% agarose gel using standard procedures. The clone thus obtained was confirmed by DNA sequencing.

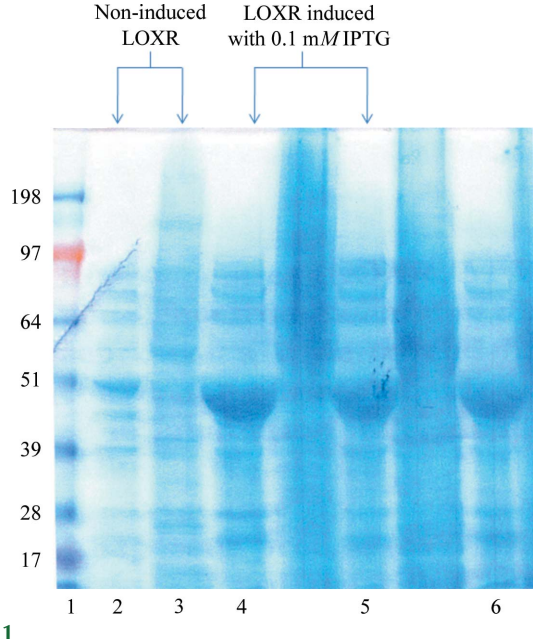


Figure 1
The expression efficiency was verified by the SDS–PAGE technique. Lane 1 contains SDS–PAGE markers (labeled in kDa). Lane 2 contains non-induced protein. Lanes 4–6 contain protein (LOXR with chaperone) induced with IPTG.

2.3. Expression of LOX protein

LOXR/pET15b vector and chaperone plasmid PG-Tf2 were co-transformed into BL21 (DE3) cells. Transformed cells were selected for growth on LB agar plates supplemented with 50 µg ml^{-1} carbenicillin and 50 µg ml^{-1} chloramphenicol. An individual colony was selected to inoculate 200 ml LB medium containing 50 µg ml^{-1} carbenicillin and 50 µg ml^{-1} chloramphenicol. The culture was incubated at 310 K with constant shaking overnight. 200 ml overnight culture was then added to a litre of LB with 50 µg ml^{-1} carbenicillin and 50 µg ml^{-1} chloramphenicol and incubated at 310 K to an OD_{600} of 0.15. At this point 10 µg ml^{-1} tetracycline was added to the culture to express chaperone plasmid PG-Tf2 proteins. The culture was incubated at 288 K until an OD_{600} of 0.4 was reached. The culture was induced with 1 m*M* IPTG and incubated at 288 K with shaking for 24 h. After 24 h, the cells were harvested by centrifugation at 15 000 rev min^{-1} at 277 K for 10 min. The expression efficiency was verified by the SDS–PAGE technique (Fig. 1).

2.4. Purification of the 12R-LOX–chaperone complex

The cell pellet containing expressed 12R-LOX was resuspended in 100 ml lysis buffer (50 m*M* Tris pH 8.0, 500 m*M* NaCl, 10 m*M* imidazole, 1% Triton X-100, 5 m*M* β-mercaptoethanol, 10% glycerol with two protease-inhibitor tablets). The resuspended cells were incubated on ice, sonicated for 10 min and centrifuged at 20 000 rev min^{-1} for 22 min. After centrifugation, the supernatant was loaded onto an immobilized Ni^{2+} metal-affinity chromatography (IMAC) column equilibrated with buffer *A* [50 m*M* Tris pH 8.0, 500 m*M* NaCl, 5 m*M* β-mercaptoethanol, 10% glycerol, 1 m*M* imidazole] and eluted with an increasing concentration of buffer *B* [50 m*M* Tris pH 8.0, 500 m*M* NaCl, 5 m*M* β-mercaptoethanol, 10% glycerol, 500 m*M* imidazole]. The purity of the protein was tested by SDS–PAGE. SDS–PAGE showed that bands containing the LOXR–chaperone complex eluted from the IMAC column at 50, 100, 250 and 500 m*M* imidazole. MALDI–TOF mass-spectrometric analysis indicated that the higher band running at approximately 58 kDa was the chaperone protein GroEL. These two proteins could not be separated, although a number of conventional chromatographic methods were attempted, including ion exchange, hydroxyapatite, hydrophobic interaction and gel filtration. High salt concentrations have been shown to disrupt protein–protein interactions. Fractions containing 12R-LOX and GroEL were pooled and concentrated to 3 ml.

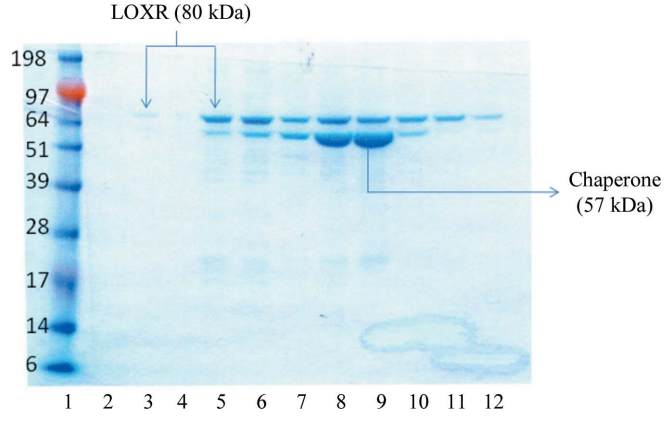


Figure 2
12% SDS–PAGE gel of the LOXR–chaperone complex obtained from a Superdex 200 gel-filtration column. Lane 1 contains molecular-mass markers (LMW, Pharmacia; labeled in kDa). Lanes 6–10 contain fractions containing LOXR with chaperone from the gel-filtration column.

At this point, 2 *M* NaCl was added and the protein was incubated overnight at 277 K. After affinity-column purification, fractions containing 12R-LOX were pooled together and concentrated to 3 ml. 2 *M* NaCl was added and the protein was again incubated overnight at 277 K. The next day, the protein was loaded onto a gel-filtration column (Superdex 200) equilibrated with 50 m*M* Tris pH 8.0, 1 *M* NaCl and 5 m*M* β-mercaptoethanol. Fractions from the gel-filtration column were analyzed by SDS–PAGE (Fig. 2). Fractions from the gel-filtration column were pooled together and concentrated to 5 ml for thrombin digestion overnight at room temperature. Digested and undigested proteins were separated by IMAC for purification. Both digested and undigested proteins were concentrated to 1 ml for dialysis against 50 m*M* Tris pH 8.0, 50 m*M* NaCl, 5 m*M* β-mercaptoethanol at 277 K. The native gel (6% acrylamide) in Fig. 3 shows LOXR protein bound to chaperone protein. After concentration of the protein to 20 mg ml^{-1}, ligand was added to the protein solution in a 1:1 molar ratio and it was set up for crystallization.

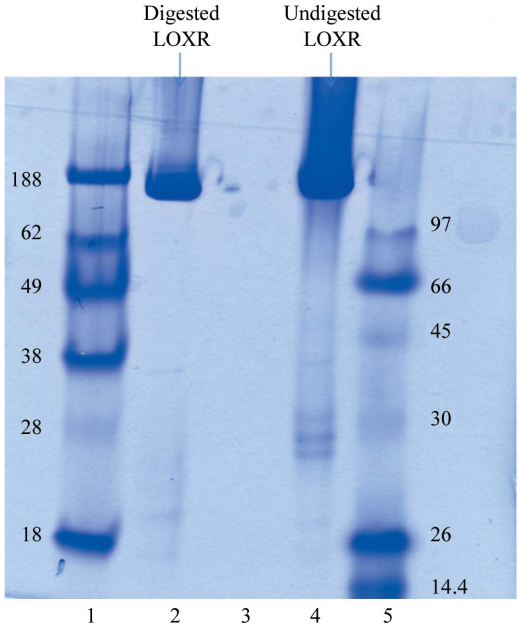


Figure 3
A native gel showing LOXR protein bound to chaperone. Lane 1 contains SDS–PAGE high-molecular-weight marker (labeled in kDa). Lane 2 contains digested LOXR protein bound to chaperone. Lane 4 contains undigested LOXR protein bound to chaperone. Lane 5 contains SDS–PAGE low-molecular-weight marker (labeled in kDa).

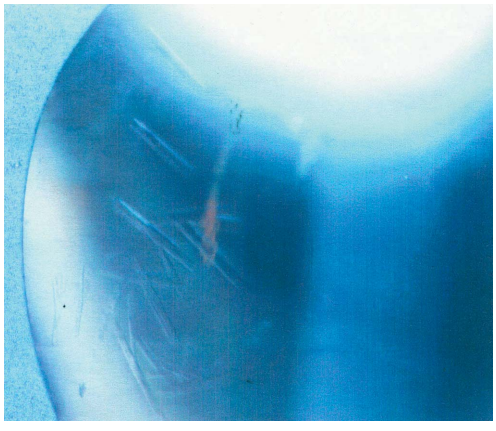


Figure 4
Needle-shaped crystals of the LOXR–chaperone complex with ligand.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Wavelength (Å)	1.5418
Unit-cell parameters (Å, °)	a = 138.97, b = 266.11, c = 152.26, β = 101.07
Temperature (K)	100
Mosaicity (°)	1.4
Exposure time (s)	150
Oscillation range (°)	1
Resolution (Å)	29–3.1 (3.27–3.10)
Measured/unique reflections	170994/85165 (18031/6786)
Completeness (%)	94.1 (87.8)
Mean $I/\sigma(I)$	6.5 (2.4)
Average multiplicity	4.3 (2.6)
$R_{\rm merge}$† (%)	8.4 (22.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity for the ith measurement of reflection hkl, $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl for all i measurements, $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

2.5. Crystallization

We obtained several crystals of the LOXR–chaperone complex with the ligand arachidonic acid as a substrate of the enzyme. Initially, crystallization conditions were screened by the sparse-matrix method (Jancarik & Kim, 1991) using the Crystal Screen and Crystal Screen 2 kits (Hampton Research, USA). Typically, 2 µl protein solution at a concentration of 20 mg ml^{-1} was mixed with an equal volume of reservoir solution and equilibrated at 293 K against 1 ml reservoir solution. The complete process of crystallization was performed by the hanging-drop vapor-diffusion method (Ducruix & Giegé, 1992) using Linbro multiwell tissue-culture plates. Crystallization trials showed needle-shaped crystals (Fig. 4). Subsequent optimization refined the crystallization conditions; the optimized composition of the precipitant was found to be 1.5 *M* ammonium hydrogen phosphate in 0.1 *M* Tris–HCl buffer pH 8.0. Under the optimized conditions, crystals appeared in 7 d and reached maximum dimensions that were suitable for X-ray diffraction.

2.6. X-ray diffraction data collection and preliminary data analysis

The best crystal, with dimensions of 0.2 × 0.02 × 0.04 mm, was used for data collection. Prior to data collection, the crystal was soaked for several seconds in a solution containing 30% MPD as a cryoprotectant and was flashed-cooled to 100 K in a liquid-nitrogen cryostream. X-ray diffraction data collection was performed at 100 K using synchrotron radiation on beamline X9B at Brookhaven National Laboratory, USA. The crystal diffracted to 4 Å resolution and X-ray data were collected from a single crystal using a crystal-to-detector distance of 350 mm. X-ray diffraction data were indexed and processed using *DENZO*, *SCALEPACK* and programs from the *CCP*4 suite (Otwinowski & Minor, 1997; Winn *et al.*, 2011). Data-collection statistics are given in Table 1. The crystals belonged to the monoclinic system with space group $P2_1$ and the unit-cell parameters were found to be a = 138.97, b = 266.11, c = 152.26 Å, β = 101.07°. It has been found that the chaperone protein GroEL (57.16 kDa) remains as a heptamer (Hemmingsen *et al.*, 1988) and two such heptamer rings encapsulate LOXR, giving a molecular weight for the complex of 880.58 kDa. With one complex molecule in the asymmetric unit, the Matthews coefficient (Matthews, 1968) was calculated to be 3.1 Å^3 Da^{-1} and the solvent content was 60.5%. We were able

to collect X-diffraction data to 4 Å resolution using synchrotron radiation and it is planned to solve the structure by the molecular-replacement method utilizing the *E. coli* GroEL structure as a search model.

3. Results and discussion

Expression of human lipoxygenase with chaperone revealed many important differences with respect to stability and purification. The focus of the work was to produce sufficient quantities of lipoxygenase protein and crystallize it with the ligand arachidonic acid for determination of the three-dimensional structure by the X-ray diffraction method. Based on the three-dimensional X-ray crystal structures of soybean lipoxygenases and rabbit reticulocyte 15-lipoxygenase, members of the lipoxygenase family possess two domains: a short barrel N-terminal domain of unknown function and a major catalytic domain that includes the nonheme Fe atom. Gillmor *et al.* (1997) have hypothesized that the barrel N-terminal region, which has homology to lipoprotein lipase, may participate in binding lipid membranes to gain access to the source of substrate. In the case of 5-lipoxygenase, they suggested a possible site of interaction with 5-lipoxygenase-activating protein, a co-accessory protein in leukotriene biosynthesis that may help to 'transfer' arachidonic acid substrate to the enzyme (Chandrasekharan *et al.*, 2002). We cloned *E. coli* lipoxygenase, overexpressed it with chaperone and purified it to homogeneity. The overexpressed protein complexed with chaperone was produced in a highly soluble form. The purity of the protein was determined by mass spectrometry. The expression and purification procedures in the study have provided a simple and efficient method to obtain pure *E. coli* lipoxygenase in large quantities. The single-step purification by affinity chromatography was sufficient to produce crystallization-quality protein. Crystal growth usually occurred in 7–10 d, leading to needle-shaped crystals with dimensions of 100–400 µm. Crystals were obtained in more than ten different conditions containing ammonium hydrogen phosphate as precipitant in the pH range 6.0–8.0. Refinement of the crystallization conditions revealed that crystals grown using 1.5 *M* ammonium hydrogen phosphate in 0.1 *M* Tris–HCl pH 8.0 were of diffraction quality and diffracted X-rays to 4 Å resolution. We are hopeful that the structure of full-length LOXR will provide information on the N-terminal domain and the catalytic domain. Here, we present the soluble expression of LOXR with a molecular chaperone and its purification, crystallization and preliminary X-ray diffraction analysis in order to determine the crystal structure of the LOXR–chaperone complex and the mode of interaction of the LOXR protein with its ligand.

The research was supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health. The authors gratefully acknowledge Brookhaven National Laboratory for the provision of beamline and other facilities.

References

Baer, A. N. & Green, F. A. (1993). *J. Lipid Res.* **34**, 1505–1514.
Brash, A. R. (1999). *J. Biol. Chem.* **274**, 23679–23682.
Camp, R. D., Mallet, A. I., Woollard, P. M., Brain, S. D., Black, A. K. & Greaves, M. W. (1983). *Prostaglandins*, **26**, 431–447.
Cao, Y. & Prescott, S. M. (2002). *J. Cell. Physiol.* **190**, 279–286.
Chandrasekharan, N. V., Dai, H., Roos, K. L., Evanson, N. K., Tomsik, J., Elton, T. S. & Simmons, D. L. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 13926–13931.
Ducruix, A. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins: A Practical Approach*. Oxford University Press.
Gillmor, S. A., Villaseñor, A., Fletterick, R., Sigal, E. & Browner, M. F. (1997). *Nature Struct. Biol.* **4**, 1003–1009.
Hammarström, S., Hamberg, M., Samuelsson, B., Duell, E. A., Stawiski, M. & Voorhees, J. J. (1975). *Proc. Natl Acad. Sci. USA*, **72**, 5130–5134.
Hemmingsen, S. M., Woolford, C., van der Vic, S. M., Tilly, K., Dennis, D. T., Georgopou, C. P., Hendrix, R. W. & Ellis, R. J. (1988). *Nature (London)*, **333**, 330–334.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jobard, F., Lefèvre, C., Karaduman, A., Blanchet-Bardon, C., Emre, S., Weissenbach, J., Ozgüc, M., Lathrop, M., Prud'homme, J. F. & Fischer, J. (2002). *Hum. Mol. Genet.* **11**, 107–113.
Krieg, P., Marks, F. & Fürstenberger, G. (2001). *Genomics*, **73**, 300–323.
Kühn, H. & Borngräber, S. (1999). *Adv. Exp. Med. Biol.* **447**, 5–28.
Kühn, H., Saam, J., Eibach, S., Holzhütter, H. G., Ivanov, I. & Walther, M. (2005). *Biochem. Biophys. Res. Commun.* **338**, 93–101.
Kühn, H. & Thiele, B. J. (1999). *FEBS Lett.* **449**, 7–11.
Lee, D.-H., Kim, M.-D., Lee, W.-H., Kweon, D.-H. & Seo, J.-H. (2004). *Appl. Microbiol. Biotechnol.* **63**, 549–552.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mizobata, T., Kagawa, M., Murakoshi, N., Kusaka, E., Kameo, K., Kawata, Y. & Nagai, J. (2000). *Eur. J. Biochem.* **267**, 4264–4271.
Nishihara, K., Kanemori, M., Kitagawa, M., Yanagi, H. & Yura, T. (1998). *Appl. Environ. Microbiol.* **64**, 1694–1699.
Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T. (1999). *Appl. Environ. Microbiol.* **66**, 884–889.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Sareen, D., Sharma, R. & Vohra, R. M. (2001). *Protein Expr. Purif.* **23**, 374–379.
Schlieker, C., Bukau, B. & Mogk, A. (2002). *J. Biotechnol.* **96**, 13–21.
Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.
Woollard, P. M. (1986). *Biochem. Biophys. Res. Commun.* **136**, 169–176.