ALOX12

PDB:3D3L

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|4502051
Entry Clone Source:
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host: *E.coli* BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smLDFEWTLKAGALEMALKRVYTLLSSWNCLEDFDQIFWGQKSALAEKVRQCWQDDELFSYQFLN
GANPMLLRRSTSLPSRLVLPSGMEELRAQLEKELQNGSLFEADFILLDGIPANVIRGEKQYLAAPLVMLKMEPNGKLQPMVIQIQPP
NPSSPTPTLFLPSDPPLAWLLAKSWVRNSDFQLHEIQYHLLNTHLVAEVIAVATMRCLPGLHPIFKFLIPHIRYTMEINTRARTQLI
SDGGIFDKAVSTGGGGHVQLLRRAAAQLTYCSLCPPDDLADRGLLGLPGALYAHDALRLWEIIARYVEGIVHLFYQRDDIVKGDPEL
QAWCREITEVGLCQAQDRGFPVSFQSQSQLCHFLTMCVFTCTAQHAAINQGQLDWYAWVPNAPCTMRMPPPTTKEDVTMATVMGSLP
DVRQACLQMAISWHLSRRQPDMVPLGHHKEKYFSGPKPKAVLNQFRTDLEKLEKEITARNEQLDWPYEYLKPSCIENSVTSDSKGGY
GSEFELRRQACGRTRAPPPPPLRSGC

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 50 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (50 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl PPG P2,000 81380 anti-foam solution (Fluka). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The bottle was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (44.3 g wet cell weight) was stored at -80 °C.

Purification

Procedure

Columns
IMAC: Ni-charged 5 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 26/60 Superdex 200 Prep Grade (GE Healthcare)

Procedure
Purification of the protein was performed as a two step process on an ÄKTAxpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. The filtered lysate was loaded onto the Ni-charged HiTrap Chelating column and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were pooled and subsequently concentrated in a centrifugal filter device to 11.9 mg/ml in a volume of 1.2 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell pellet was thawed and resuspended in lysis buffer (2.0 ml/g cell pellet), supplemented with 1000 U Benzonase (Merck), a knife edge lysozyme (Sigma) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off), repeated two times. Cell debris was removed by centrifugation (49,000 x *g*, 40 min, 4 ºC) and the supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. Prior to crystallization, 2 mM FeSO4 was added to the protein solution and incubated on ice for 15 minutes. 0.2 µl of the protein solution (12 mg/ml) with FeSO4 was mixed with 0.1 µl of well solution consisting of 0.1 M citric acid pH 4, 1 M LiCl and 20% (w/v) PEG 6000. The plate was incubated at 20 ºC and plate shaped crystals appeared within three days. The crystal was quickly transferred to cryo solution consisting of well solution, 0.3 M NaCl and 20% glycerol, and flash-frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection:235° was collected by a frame of 1° on beamline BM14 at ESRF (0.976270).
Data Processing: The crystal belonged to space group P1 with the cell parameters a=59.590, b=70.153, c=77.868, α=65.37, β=88.01 and γ=69.82. Data was processed and scaled using XDS and Scala respectively. The structure was solved by molecular replacement using Molrep. The probe was a model of ALOX12 provided by Swiss-Model, based upon the structure of the rabbit reticulocyte 15-lipoxygenase (PDB entry: 1LOX). Two monomers were found in the asymmetric unit. Data in the interval 47.2 - 2.6 Å resolution was used for refinement. First steps of refinement were carried out by simulated annealing using CNS after removing the 55 first residues of the model and adding one iron per monomer. After these two steps, R and Rfree reached 32.6% and 33.1% respectively. The final model was obtained by iterative cycle of manual building in COOT and refinement in REFMAC5. At the end of the refinement the values for R and Rfree were 20.6% and 27.6% respectively.