ACOT12

PDB:3B7K

Revision

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Revised by:created
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Entry Clone Accession:gi|18640736
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:ACOT12BA-k003
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21-Gold(DE3)pRARE2, where BL21-Gold(DE3) cells (Stratagene) have been transformed with pRARE2 originating from the Rosetta2 strain (Novagen). The pRARE2 plasmid supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smGEVVMSQAIQPAHATARGELSAGQLLKWIDTTACLAAEKHAGVSCVTASVDDIQFEETARVGQ
VITIKAKVTRAFSTSMEISIKVMVQDMLTGIEKLVSVAFSTFVAKPVGKEKIHLKPVTLLTEQDHVEHNLAAERRKVRLQHEDTFNN
LMKESSKFDDLIFDEEEGAVSTRGTSVQSIELVLPPHANHHGNTFGGQIMAWMETVATISASRLCWAHPFLKSVDMFKFRGPSTVGD
RLVFTAIVNNTFQTCVEVGVRVEAFDCQEWAEGRGRHINSAFLIYNAADDKENLITFPRIQPISKDDFRRYRG

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 20 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 ºC overnight. The overnight culture (20 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~2. The culture was down-tempered to 18 ºC 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 (5,500 x *g*, 10 min, 4 ºC). The resulting cell pellet (38.8 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 ºC.

Purification

Procedure

Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 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. The chromatogram from the gel filtration column showed a symmetric peak with a retention volume approximately corresponding to a trimer. Fractions containing the target protein were pooled and fresh TCEP was added to a final concentration of 2 mM. The protein was subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 16.3 mg/ml in a volume of 1.7 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water and 2000 U Benzonase (Merck) were added. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: The crystal was obtained by the hanging drop vapour diffusion method in a 24-well plate containing 500 µl well solution. 1 µl of the protein solution (diluted to 10 mg/ml) including 5 mM acetyl-CoA, was mixed with 1 µl of well solution consisting of 0.35 M NaSCN and 25% PEG 3350. The plate was incubated at 20 ºC. The crystal was quickly transferred to cryo solution containing well solution and 5% butanediol and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data to 2.7 Å resolution was collected at ESRF beamline ID14-1. A single crystal was used to collect 180º oscillation range.
Data Processing: Data was processed with XDS in space group C2221 (a= 82.7 Å, b= 126.1 and c= 185.3 Å). The structure was solved by molecular replacement using the PDB entry 1VPM as model. Side chains of a thioesterase domain were truncated based on sequence alignment with Mr. BuMP and a dimer based on 1VPM was created with COOT. This dimer was used as a model in MOLREP, which found 3 dimers organized as a trimer of dimers. Model building was done with COOT and the structure was refined with REFMAC5.