ACOT11

PDB:3FO5

Revision

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Entry Clone Accession:BC093846
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21-Gold(DE3)pG-Tf2, denotes BL21-Gold(DE3) cells from Stratagene transformed with the chaperone coding plasmid pG-Tf2 (TaKaRa).

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smRPQPGDGERRYREASARKKIRLDRKYIVSCKQTEVPLSVPWDPSNQVYLSYNNVSSLKMLVAK
DNWVLSSEISQVRLYTLEDDKFLSFHMEMVVHVDAAQAFLLLSDLRQRPEWDKHYRSVELVQQVDEDDAIYHVTSPALGGHTKPQDF
VILASRRKPCDNGDPYVIALRSVTLPTHRETPEYRRGETLCSGFCLWREGDQLTKVSYYNQATPGVLNYVTTNVAGLSSEFYTTFKA
CEQFLLDNRNDLAPSLQTL

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:Native protein
Freshly transformed cells were used to inoculate 20 ml LB, supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, and grown at 37 °C overnight. 15 ml of the overnight culture 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. At this time chaperone expression was induced with 2 ng/ml tetracycline and the bottle was down-tempered to 18 °C over a period of 1 hour. Expression of target protein was then induced by addition of 0.5 mM IPTG and allowed to continue overnight. Cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (32.1 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet) supplemented with one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science) and 1500 U Benzonase (Merck) and stored at -80 °C.

SeMet labeled protein
Cells from glycerol stock were grown in 2 x 70 ml LB supplemented with 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. 90 ml of the overnight culture were used to inoculate 6 bottles with 1.5 l minimal medium (without amino acids) supplemented with 50 µg/ml kanamycin and approximately 500 µl Dow Corning anti-foam RD emulsion 63213 4D (BDH Silicone Products) per bottle. The cultures were grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~1. The cultures were down-tempered to 18 °C, amino acids were added and expression of chaperones was induced by addition of 2 ng/ml tetracycline. One hour later, expression of target protein was induced by addition of 0.5 mM IPTG. The expression was allowed to continue at 18 °C overnight. Cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (79 g from 9 liter culture) was resuspended in lysis buffer (1ml/g cell pellet) supplemented with 6 tablets of Complete EDTA-free protease inhibitor (Roche Applied Science) and 12000 U Benzonase (Merck) and stored at -80 °C.
Composition of Minimal media: 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH4Cl, 5 mM Na_2SO4, 0.4% (w/v) glucose, 2mM MgSO4, 0.1mM CaCl2, 1.0 µM MnCl2, 10 µM FeSO4.
Mix of amino acids added per liter culture (Van Duyne, G. D., *J. Mol. Biol.* **229**, 105-124 (1993)): 100 mg each of lysine, threonine, phenylalanine and 50 mg each of leucine, isoleucine, valine, L(+)-selenomethionine.

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 native 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 into two samples and subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore). The concentration was measured to 4.3/5.7 mg/ml in a volume of 2 x 0.3 ml and the samples were stored at -80 ºC. Purification of the SeMet enriched protein was performed in basically the same way. The filtered lysate was purified by IMAC and gel filtration chromatography followed by a concentration step. The final SeMet-protein concentration was 11.6 mg/ml in a volume of 6.5 ml.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating native protein (5.7 mg/ml in 0.3 ml) with His-tagged TEV protease at a molar ratio of 20:1. The proteolytic reaction went to completion, as judged by SDS-PAGE. The volume of the reaction mixture was adjusted to approximately 1.5 ml with GF buffer containing 20 mM imidazole. The target protein was subsequently purified from tag and protease by passing it over a Ni-charged 1 ml HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with GF buffer containing 20 mM imidazole. The cleaved protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a centrifugal filter device. The final protein concentration was determined to 11.3 mg/ml in a volume of 0.1 ml. The identity of the protein was confirmed by mass spectrometry. The histidine tag removal of SeMet labeled protein was achieved in basically the

same way as described for the native protein. Purified SeMet labeled protein (11.6 mg/ml) was incubated with TEV protease at 4 ºC over the weekend, passed over a Ni-charged 1 ml HiTrap Chelating HP column and concentrated to a final concentration of 24.1 mg/ml in 0.9 ml.

Extraction

Procedure
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (VibraCell, Sonics) at 70% amplitude for 3 min effective time (puls 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 30 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Native crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl of the protein solution (11.3 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M bis-Tris pH 5.5, 0.2 M magnesium chloride, 25% (w/v) PEG 3350. The plate was incubated at 4 ºC and crystals were obtained after 7 days. The crystals were quickly transferred to cryo solution containing well solution and 20% glycerol and flash frozen in liquid nitrogen. SeMet crystals were produced in the same way by mixing 0.2 µl of the SeMet-protein solution (diluted with GF buffer to 15 mg/ml) with 0.4 µl of well solution consisting of 0.2 M sodium thiocyanate, pH 6.9 and 20% (w/v) PEG 3350. The plate was incubated at 4 ºC and crystals were obtained after 2 days. The crystals were quickly transferred to cryo solution containing 0.2 M sodium thiocyanate, 22% (w/v) PEG 3350, 0.2 M NaCl and 18% glycerol and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data was collected from two crystals. MAD data was collected to 2.6 Å at BESSY (BL14-2) Berlin, Germany. The crystal belonged to space group P31 with cell parameters of a=b=69.4 Å and c=97.3 Å. Native data to 2.0 Å resolution was collected at ESRF (ID14-2), Grenoble, France. Native data that was used for the final refinement belonged to C 2 2 21 space group with cell parameters of a= 52.44.Å, b= 130.08 Å, c= 165.23 Å.
Data Processing: The structure was solved using Solve with the three-wavelength MAD data. Resolve was used to build the initial model, which was then improved by manual editing and PHENIX autobuild. The asymmetric unit consisted of two chains and one of them was used as a model in Molrep using the native data. The model was further improved by 3 rounds of manual model building and arp-warp. The structure was refined with Refmac5. Final R-values were R= 20.22% and Rfree= 25.12% and coordinates and structure factors were deposited in the PDB with accession code 3FO5.