CSAD

PDB:2JIS

Revision

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Entry Clone Accession:gi|68226687
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)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*smADSEALPSLAGDPVAVEALLRAVFGVVVDEAIQKGTSVSQKVCEWKEPEELKQLLDLELRSQG
ESQKQILERCRAVIRYSVKTGHPRFFNQLFSGLDPHALAGRIITESLNTSQYTYEIAPVFVLMEEEVLRKLRALVGWSSGDGIFCPG
GSISNMYAVNLARYQRYPDCKQRGLRTLPPLALFTSKECHYSIQKGAAFLGLGTDSVRVVKADERGKMVPEDLERQIGMAEAEGAVP
FLVSATSGTTVLGAFDPLEAIADVCQRHGLWLHVDAAWGGSVLLSQTHRHLLDGIQRADSVAWNPHKLLAAGLQCSALLLQDTSNLL
KRCHGSQASYLFQQDKFYDVALDTGDKVVQCGRRVDCLKLWLMWKAQGDQGLERRIDQAFVLARYLVEEMKKREGFELVMEPEFVNV
CFWFVPPSLRGKQESPDYHERLSKVAPVLKERMVKEGSMMIGYQPHGTRGNFFRVVVANSALTCADMDFLLNELERLGQDL

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:Native protein
Cells from glycerol stock were grown in 200 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 ºC overnight. 100 ml of the overnight culture was used to inoculate 4 x 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) per bottle. The cultures were grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~2.4. The cultures were 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 (105 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 4 tablets of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 ºC.

SeMet enriched protein
Cells from glycerol stock were streaked onto LB-agar plates. 5-10 colonies were used to inoculate 2 x 40 ml LB supplemented with 100 µg/ml kanamycin and 34 µg/ml chloramphenicol. The pre-cultures were grown at 30 ºC overnight. 60 ml of the overnight cultures were used to inoculate 6 bottles with 1.5 l minimal medium (without amino acids) supplemented with 50 µg/ml kanamycin, 34 µg/ml chloramphenicol and approximately 200 µl BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd) per bottle. The cultures were grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~0.8. The cultures were down-tempered to 18 ºC and amino acids were added after 30 minutes. After additional one hour, expression of target protein was induced by addition of 0.5 mM IPTG and the expression was allowed to continue at 18 ºC overnight. Cells were harvested the following morning by centrifugation (5,500 x *g*, 15 min, 4 ºC). The resulting cell pellet (44 g from 9 liter culture) was resuspended in lysis buffer (2ml/g cell pellet) supplemented with 1 tablet of Complete EDTA-free protease inhibitor (Roche Applied Science) and stored at -80 ºC. Selenomethionine labeled protein was grown in minimal medium containing: 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 0.4% (w/v) glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1.0 µM $MnCl_2$, 10 µM $FeSO_4$.
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 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 13.6 mg/ml in a volume of 0.05 ml. Purification of the SeMet enriched protein was performed in basically the same way. The filtered lysate was purified by IMAC and gel filtration followed by a concentration step. The final SeMet-protein concentration was 14 mg/ml in a volume of 0.12 ml. The identities and selenomethionine incorporation of the proteins were confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water and 8000 U Benzonase (Merck) was added. Cells were disrupted by sonication (VibraCell, Sonics) at 80% amplitude for 3 min effective time (puls 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. The SeMet-labeled protein was teated in basically the same way.
Concentration:
Ligand
MassSpec:
Crystallization: Native crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. The protein solution (diluted to 6.9 mg/ml) including 3 mM pyridoxal-5Â□-phosphate (PLP) was mixed with well solution consisting of 0.2 M ammonium nitrate and 20% (w/v) PEG 3350. The plate was incubated at 20 ºC. Crystals were obtained within 1 day and continued to grow for two more days to reach their maximal size (approx. 100 µm × 40 µm × 40 µm). The crystals were quickly transferred to cryo solution containing 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 (14 mg/ml) including 3 mM pyridoxal-5Â□-phosphate (PLP) with 0.1 µl of the well solution consisting of 0.1 M Tris pH 8.5, 0.2 M ammonium acetate and 25% (w/v) PEG 3350. The plate was incubated at 20 ºC. Crystals were obtained after three days and continued to grow for one more week. The crystals were quickly transferred to cryo solution containing glycerol and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data was collected at ESRF beamline ID14-4.
Data Processing: The structure was solved by SAD using Seleno-methionine labeled protein crystals. Diffraction data up to 1.8 Å resolution was collected. The space group was C2 with cell dimensions a=95.2 Å, b=70.6 Å, c=68.9 Å, and beta=104.2°. The asymmetric unit contained one monomer. The obtained model was thereafter used as an initial model for refinement with native data to a resolution of 1.6 Å. Here the space group was P212121 with cell dimensions a=65.3 Å, b=100.6 Å, c=163.1 Å. Two monomers were located in the asymmetric unit. Automatic model building using Arp/warp was performed; Refmac5 was used for refinement and Coot for model building. Data in the interval 39.9-1.60 Å resolution was used and at the end of the refinement the R values were: R= 15.3% and Rfree= 17.9%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2JIS.