TBSAH

PDB:3H9U

Revision

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Entry Clone Accession:gi|71756217
Entry Clone Source: In house cloning
SGC Clone Accession:TBSAHA-k011
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*smYKVRDISLAEWGRRELELAENEMPGLMELRREYGPSKPLKGAKIAGCLHMTMQTAVLIETLVE
LGAEVRWASCNIFSTQDHAAAAIAKRGIPVFAWKGETEEEYMWCMKQTLKGFSGDGYPNMLLDDGGDLTNYVLDECKELDGKIYGVS
EETTTGVKNLYKRLQRGKLTIPAMNVNDSVTKSKFDNLYGCRESLVDGIKRATDVMIAGKTACVCGYGDVGKGCAAALRGFGARVVV
TEVDPINALQAAMEGYQVLLVEDVVEEAHIFVTTTGNDDIITSEHFPRMRDDAIVCNIGHFDTEIQVAWLKANAKERVEVKPQVDRY
TMANGRHIILLAEGRLVNLGCASGHPSFVMSNSFCNQVLAQIELWTNRDTGKYPRGAKAQVYFLPKKLDEKVAALHLGKLGAKLTKL
TPKQAEYINCPVDGPFKPDHYRY

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 °C overnight. The overnight culture was used to inoculate 0.75 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture 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 (30 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 2000 U Benzonase (Merck) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

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. 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 25.2 mg/ml in a volume of 1.6 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water. 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: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.4 µl protein solution (24.7 mg/ml) was mixed with 0.2 µl of well solution consisting of 0.1 M Tris pH 8.5, 0.2 M trimethylamine n-oxide and 20% PEGMME 2000. The plate was incubated at 4 ºC and crystals appeared in two days. The crystals were quickly transferred to a cryo solution consisting of well solution complemented with 2% PEGMME 2000, 18% glycerol and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 1.9 Å resolution was collected at ESRF beamline ID23-1
Data Processing: The structure was solved by molecular replacement using PHASER with search model PDB ID = 3G1U . The space group was C 1 2 1 with cell dimensions a=91.35 Å b=136.05 Å c=191.87 Å, β=99.32°. Four monomers were located in the asymmetric unit. Pseudotranslation was observed with fraction estimated to 28%. Refinement was performed using REFMAC5 TLS refinement with TLS groups determined by the TLS Motion Determination home (http://skuld.bmsc.washington.edu/~tlsmd/). Coot was used for manual model building. Data in the interval 45.55-1.90 Å resolution was used and at the end of the refinement the R values were: R=19.8% and Rfree=23.2%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3H9U.