SPG7

PDB:2QZ4

Revision

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Entry Clone Accession:gi|62020635
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*smGVSFKDVAGMHEAKLEVREFVDYLKSPERFLQLGAKVPKGALLLGPPGCGKTLLAKAVATEAQ
VPFLAMAGAEFVEVIGGLGAARVRSLFKEARARAPCIVYIDEIDAVGKKRSTTMSGFSNTEEEQTLNQLLVEMDGMGTTDHVIVLAS
TNRADILDGALMRPGRLDRHVFIDLPTLQERREIFEQHLKSLKLTQSSTFYSQRLAELTPGFSGADIANICNEAALHAAREGHTSVH
TLNFEYAVERVLAGTAKKSKILSK

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Freshly transformed cells were used to inoculate 40 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 37 ºC overnight. The overnight culture (40 ml) was used to inoculate 2 x 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin, 34 µg/ml chloramphenicol and approximately 500 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 ~1.3. 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 (4,700 x *g*, 10 min, 4 ºC). The resulting cell pellet (64.2 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet) supplemented with two tablets of Complete EDTA-free protease inhibitor (Roche Applied Science) and 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 on an ÄKTAprime system (GE Healthcare). The filtered lysate was divided into two halves and loaded onto two separate HiTrap Chelating columns, pre-equilibrated with IMAC wash1 buffer. The columns were washed with IMAC wash1 buffer followed by IMAC wash2 buffer and the protein was eluted with IMAC elution buffer. Due to precipitation, the samples were diluted in GF-buffer and 1-10 mM of ATP and MgCl2 was added.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating approximately 25% of the protein sample (~8 mg/ml in 5 ml) with His-tagged TEV protease at a molar ratio of 50:1 at 20 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Prior to purification, columns were equilibrated with IMAC wash1 buffer (without imidazole) and gel filtration buffer, respectively. Target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged HiTrap Chelating HP column (GE Healthcare). The protein was recovered from flow through 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) and loaded onto the gel filtration column. Fractions containing the target protein were pooled and concentrated to 31.2 mg/ml in a volume of 0.2 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed and 4000 U Benzonase (Merck), 1 mM ATP and 1 mM MgCl2 was 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 (49,100 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. 0.2 µl protein solution (diluted to 20 mg/ml) including 2.5 mM ATP and 2.5 mM MgCl2, was mixed with 0.1 µl of well solution consisting of 0.1 M bis-Tris pH 5.5, 0.2 M ammonium acetate and 25% PEG 3350. The plate was incubated at 20 ºC and crystals appeared within 12 days. The crystals were quickly transferred to a cryo solution consisting of well solution complemented with 15% glycerol, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data was collected at beamline ID 14-2 at the ESRF synchrotron radiation facility in Grenoble, France. Data was indexed and integrated in space group P4322 with the XDS package.
Data Processing: The structure was solved by MR using PDB entry: 2CE7 - metalloprotease FtsH from *Thermotoga maritima* with Molrep. The asymmetric unit contains one protein monomer. The cell dimensions are a=b=57.20 Å c=155.22 Å. Refmac5 was used for refinement and Coot for model building. TLS restrained refinement using 3 TLS groups was used in the refinement process. The TLS groups were selected using the tlsmd server

http://skuld.bmsc.washington.edu/~tlsmd/. Data in the interval 39.13-2.22Å resolution was used and at the end of the refinement the R values were: R= 20.9% and Rfree= 26.2%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2QZ4.