NVL

PDB:2X8A

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:gi|15082383
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:NVLA-k018
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21(DE3) R3 pRARE 2, where the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

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mhhhhhhssgvdlgtenlyfq*smTVPNVTWADIGALEDIREELTMAILAPVRNPDQFKALGLVTPAGVLLAGPPGCGKTLLAKAVA
NESGLNFISVKGPELLNMYVGESERAVRQVFQRAKNSAPCVIFFDEVDALCPRRSDRETGASVRVVNQLLTEMDGLEARQQVFIMAA
TNRPDIIDPAILRPGRLDKTLFVGLPPPADRLAILKTITKNGTKPPLDADVNLEAIAGDLRCDCYTGADLSALVREASICALRQEMA
RQKSGNEKGELKVSHKHFEEAFKKVRSSISKKDQI
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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 (20 ml) was used to inoculate 1.5 l TB supplemented with 35 µg/ml chloramphenicol, 50 µg/ml kanamycin and approximately 10 drops PPGP2000(anti-foam solution). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD_{600} 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 (5 000 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 1000 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

IMAC columns were equilibrated with IMAC wash1 buffer, and gel filtration columns were equilibrated with GF buffer. Purification of the protein was performed on an ÄKTAxpress system (GE Healthcare). 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 identified by SDS-PAGE, pooled, and fresh TCEP was added to a final concentration of 2 mM. The protein was concentrated using an Amicon Ultra-15 centrifugal filter device (10,000 NMWL; Millipore) to 2.2 mg/ml in a volume of 16.50 ml. The protein was send for Tag removal by TEV protease.

Tag removal

The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease in a molar ratio of 30:1 at 4 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was retrieved from flow through.The protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a Vivaspin 20 centrifugal filter device with 10,000 MWCO (Sartorius). The final protein concentration was determined to 21.4 mg/ml in a volume of 0.16 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). 1 mM of ADP and 1 mM of MgCl2 was added at sonication. 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 Corning 3550 96-well 3-drop plate. The 21.4 mg/ml protein solution was mixed with 10mM MgCl2 and 10mM AMP and therefore diluted into 19.6 mg/ml. 0.2 µl of protein solution (19.6 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M Tris pH 8.6, 0.2 M lithium sulfate and 40% PEG 400. The plate was incubated at 20 ºC and crystals appeared within 1 day. The crystals were flash frozen in liquid nitrogen without additional cryoprotection.
NMR Spectroscopy:
Data Collection: Diffraction data to 2.6 Å resolution was collected at DIAMOND, beamline I02.
Data Processing: The structure was solved by molecular replacement using the CaspR server. The space group was $P6_1$ with cell dimensions a=b=78.0 Å c=86.0 Å. A single monomer located in the asymmetric unit. Merohedral twinning was observed, however the twin fraction was estimated to 8% (small) and therefore twinning was neither consided in selecting the Rfree set

nor during refinement. Refmac5 and finally autoBUSTER was used for refinement and Coot for model building. Data in the interval 17.8-2.6 Å resolution was used and at the end of the refinement the R values were: R=21.4% and Rfree=25.0%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2X8A.