DDX47

PDB:3BER

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:BC068009
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:

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mhhhhhhssgvdlgtenlyfq*smEEHDSPTEASQPIVEEEETKTFKDLGVTDVLCEACDQLGWTKPTKIQIEAIPLALQGRDIIGL
AETGSGKTGAFALPILNALLETPQRLFALVLTPTRELAFQISEQFEALGSSIGVQSAVIVGGIDSMSQSLALAKKPHIIIATPGRLI
DHLENTKGFNLRALKYLVMDEADRILNMDFETEVDKILKVIPRDRKTFLFSATMTKKVQKLQRAALKNPVKCAVSS
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Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were streaked onto LB-agar plates. 5-10 colonies were used to inoculate 20 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol and the cells were grown at 30 ºC overnight. The overnight culture (20 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin, 34 µg/ml chloramphenicol and approximately 250 µl PPG P2,000 81380 anti-foam solution (Fluka). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~1.2. 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 (5,500 x *g*, 15 min, 4 ºC). The resulting cell pellet (18.9 g wet cell weight) was resuspended in lysis buffer (1 ml/g cell pellet), supplemented with 0.5 tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 ºC.

Purification

Procedure

Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 75 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 a Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 17.6 mg/ml in a volume of 0.13 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water and 1000 U Benzonase (Merck), 2 mM ADP and 2 mM MgCl2 were added to the cell suspension. The cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off). Cell debris was removed by centrifugation (49,100 x *g*, 20 min, 4 ºC) and 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. 0.1 µl protein solution (diluted to 17 mg/ml) including 5 mM ADP and 5 mM MgCl2 was mixed with 0.2 µl of well solution consisting of 0.1 M Na/K-phosphate pH 5.2, 0.05 M NaCl and 42.5% (v/v) PEG 200. The plate was incubated at 4 ºC and one pyramidal crystal appeared after three weeks and continued to grow for two more weeks to reach maximal size (approx. 200 µm × 140 µm × 30 µm). The crystal was picked directly from the drop and flash frozen in liquid nitrogen without cryo protectant.
NMR Spectroscopy:
Data Collection: Diffraction data up to 1.4 Å resolution was collected at ESRF beamline ID-29.
Data Processing: The structure was solved by molecular replacement using HERA N-terminal domain as template (PDB: 2GXS). The space group was C2 with cell dimensions a=93.05 Å b=70.37 Å c=35.86 Å. One monomer was 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 30.0-1.40 Å resolution was used and at the end of the refinement the R values were: R=16.54% and Rfree=18.91%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3BER.