DDX19

PDB:3G0H

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:BC003626
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(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:

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mhhhhhhssgvdlgtenlyfq*smEDRAAQSLLNKLIRSNLVDNTNQVEVLQRDPNSPLYSVKSFEELRLKPQLLQGVYAMGFNRPS
KIQENALPLMLAEPPQNLIAQSQSGTGKTAAFVLAMLSQVEPANKYPQCLCLSPTYELALQTGKVIEQMGKFYPELKLAYAVRGNKL
ERGQKISEQIVIGTPGTVLDWCSKLKFIDPKKIKVFVLDEADVMIATQGHQDQSIRIQRMLPRNCQMLLFSATFEDSVWKFAQKVVP
DPNVIKLKREEETLDTIKQYYVLCSSRDEKFQALCNLYGAITIAQAMIFCHTRKTASWLAAELSKEGHQVALLSGEMMVEQRAAVIE
RFREGKEKVLVTTNVCARGIDVEQVSVVINFDLPVDKDGNPDNETYLHRIGRTGRFGKRGLAVNMVDSKHSMNILNRIQEHFNKKIE
RLDTDDLDEIE
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Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 30 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. 10 ml of the overnight culture was used to inoculate 750 ml TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 100 µl PPG P2,000 81380 anti-foam solution (Fluka). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2.2. The bottle 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 (14 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet), supplemented with 1250 U Benzonase (Merck) and 0.5 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 with 30,000 NMWL (Millipore) to 20.6 mg/ml in a volume of 1 ml.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease (van den Berg, S., *J. Biotech* **121**, 291-298 (2006)) at a molar ratio of 30:1 at 4 ºC over the weekend. 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 HisTrap HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was eluted at 35 mM imidazole.The cleaved protein was concentrated using a Vivaspin 20 centrifugal filter device with 30,000 MWCO (Sartorius) and subsequently dialyzed against GF buffer containing 2 mM TCEP. The final protein concentration was determined to 27.5 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). 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. Protein solution, deca-uracil ssRNA, ADPNP and MgCl2 were mixed and concentrated to yield a final sample composition of 20 mg/ml protein, with a 10 molar excess of decauracil ssRNA, ADPNP and MgCl2 in 30 mM HEPES, 10% glycerol, 50 mM NaCl, pH 7.5. 0.1 µl of the protein sample was then mixed with 0.2 µl of well solution consisting of 0.1 M Tris pH 8, 0.25 M trimethylamine n-oxide, 14% PEG MME 2000. The plate was incubated at 4 ºC and crystals appeared after seven days. Cryo solution containing well solution and 25% glycerol was added directly to the drop. Crystals were mounted and flash-frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data sets were collected at ESRF (beamline ID-29) to 2.7 Å resolution. The crystal belonged to space group P212121 with cell dimensions a=41.6 Å, b=81.1 Å, c=124.7 Å.

One monomer was located in the asymmetric unit.
Data Processing: Data were integrated and scaled using XDS. The structure was solved by Phaser using the DDX19 apo-structure (3ews) as a search model. Domains were searched for separately. The structure was refined with RefMac5. TLS parameters were refined using individual domains as rigid groups. Model building was done using Coot. The structure finally refined to R-factors of 0.220 and 0.275 for R and Rfree, respectively. Coordinates were deposited in the Protein Data Bank with an accession code 3g0h.