DDX10

PDB:2PL3

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|13514831
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*smQVERESISRLMQNYEKINVNEITRFSDFPLSKKTLKGLQEAQYRLVTEIQKQTIGLALQGKDV
LGAAKTGSGKTLAFLVPVLEALYRLQWTSTDGLGVLIISPTRELAYQTFEVLRKVGKNHDFSAGLIIGGKDLKHEAERINNINILVC
TPGRLLQHMDETVSFHATDLQMLVLDEADRILDMGFADTMNAVIENLPKKRQTLLFSATQTKSVKDLARLSLKNPEYVWVHEKA
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Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were streaked onto a LB-agar plate. 5-10 colonies were used to inoculate 25 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol 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 and approximately 200 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 ~1.2. 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*, 10 min, 4 ºC). The resulting cell pellet (19.8 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 an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 7.3 mg/ml in a volume of 1.2 ml.

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 for one hour. The proteolytic reaction went to completion, as judged by SDS-PAGE. The target protein was diluted 1:1 in GF-buffer to reduce the TCEP concentration. The protein was purified from tag and protease by adding Ni-NTA resins to the reaction mixture, pre-equilibrated with IMAC wash1 buffer, and collecting the flow through fraction. The protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a centrifugal filter device with 10,000 MWCO. The final protein concentration was determined to 18.7 mg/ml in a volume of 0.33 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) 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 (49,000 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:18.7 mg/ml.
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 20 mM ADP and 10 mM MgCl2 was added to the protein sample and 0.1 µl of the protein solution (diluted to 16.6 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M Tris pH 8.5, 50 mM sodium chloride and 20% ethanol. The plate was incubated at 4 ºC. The crystals appeared within one day and continued to grow for one more week to reach their maximal size (approx. 160 µm × 50 µm × 50 µm). The crystal was quickly transferred to a cryo solution consisting of well solution complemented with 40% glycerol, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data was collected at MAXLab (I911-2).
Data Processing: The structure was solved by molecular replacement using Hera N-terminal domain from *Thermus thermophilus* as a search model (PDB entry: 2GXS) with the program MolRep. The asymmetric unit contains one protein monomer. The space group was P6122 with cell dimensions a=b=63.50 Å c=304.01 Å. Refmac5 was used for refinement and Coot for model building. TLS restrained refinement using 4 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 29.3-2.15 Å resolution was used and at the end of the refinement the R values were: R= 21.1% and Rfree= 24.8%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2PL3.