RRM2B

PDB:2VUX

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:NM_015713.3
Entry Clone Source: Origene
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*smSDTNESEIKSNEEPLLRKSSRRFVIFPIQYPDIWKMYKQAQASFWTAEEVDLSKDLPHWNKLK
ADEKYFISHILAFFAASDGIVNENLVERFSQEVQVPEARCFYGFQILIENVHSEMYSLLIDTYIRDPKKREFLFNAIETMPYVKKKA
DWALRWIADRKSTFGERVVAFAAVEGVFFSGSFAAIFWLKKRGLMPGLTFSNELISRDEGLHCDFACLMFQYLVNKPSEERVREIIV
DAVKIEQEFLTEALPVGLIGMNCILMKQYIEFVADRLLVELGFSKVFQAENPFDFMENISLEGKTN
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Vector:pNIC-BSA4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 10 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (10 ml) was used to inoculate 0.75 l 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. 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 (18 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 1000 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 10,000 NMWL (Millipore) to 25.3 mg/ml in a volume of 1.5 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water and diluted to 68 ml with lysis buffer. 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 using a 96-well plate. Prior to crystallization, chymotrypsin was added to the target protein in a ratio chymotrypsin:protein 1:100. 0.2 µl of the protein solution (25.3 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M Na cacodylate, pH 6.5, 2.3 M ammonium sulfate, 0.2 M NaCl. The plate was incubated at 4 ºC and crystals appeared within 2 days. The crystal was transferred to cryo solution consisting of 0.1 M Na-cacodylate, pH 6.5, 0.3 M NaCl, 2.1 M AmSO4 and 20% glycerol, and flash-frozen in liquid nitrogen. Analysis by mass spectrometry of target protein, treated with chymotrypsin overnight at room temperature, revealed a sequence most likely represented in the crystal: SDTNESE IKSNEEPLLR KSSRRFVIFP IQYPDIWKMY KQAQASFWTA EEVDLSKDLP HWNKLKADEK YFISHILAFF AASDGIVNEN LVERFSQEVQ VPEARCFYGF QILIENVHSE MYSLLIDTYI RDPKKREFLF NAIETMPYVK KKADWALRWI ADRKSTFGER VVAFAAVEGV FFSGSFAAIF WLKKRGLMPG LTFSNELISR DEGLHCDFAC LMFQYLVNKP SEERVREIIV DAVKIEQEFL TEALPVGLIG MNCILMKQYI EFVADRLLVE LGFSKVFQAE NPFDFMENIS LEGK, with a molecular weight of 35019.3 Da.
NMR Spectroscopy:
Data Collection: Data was collected at ESRF (ID23-1).
Data Processing: Crystals belong to space group P212121 with the cell parameters 68.96, 99.47, 132.58, 90.00, 90.00, 90.00 . Data was processed and scaled using XDS and XSCALE. The structure was solved by molecular replacement using MOLREP using the structure of the human RNR, R2 subunit (PDB-code: 2UW2) as search model. Simulated annealing was run in CNS, manual model building was done with COOT and the structure was refined with REFMAC5 using one TLS groups covering all protein atoms in the end of the refinement. Data in the interval 20 - 2.8 Å resolution was used and final values for R and Rfree were 20.9% and 25.8%

respectively. The coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2VUX.