RRM1

PDB:2WGH

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|4506749,BC006498
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:RRM1A-k039
Tag:C-terminal hexahistidine tag -ahhhhhh
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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MAILAARIAVSNLHKETKKVFSDVMEDLYNYINPHNGKHSPMVAKSTLDIVLANKDRLNSAIIYDRDFSYNYFGFKTLERSYLLKIN
GKVAERPQHMLMRVSVGIHKEDIDAAIETYNLLSERWFTHASPTLFNAGTNRPQLSSCFLLSMKDDSIEGIYDTLKQCALISKSAGG
IGVAVSCIRATGSYIAGTNGNSNGLVPMLRVYNNTARYVDQGGNKRPGAFAIYLEPWHLDIFEFLDLKKNTGKEEQRARDLFFALWI
PDLFMKRVETNQDWSLMCPNECPGLDEVWGEEFEKLYASYEKQGRVRKVVKAQQLWYAIIESQTETGTPYMLYKDSCNRKSNQQNLG
TIKCSNLCTEIVEYTSKDEVAVCNLASLALNMYVTSEHTYDFKKLAEVTKVVVRNLNKIIDINYYPVPEACLSNKRHRPIGIGVQGL
ADAFILMRYPFESAEAQLLNKQIFETIYYGALEASCDLAKEQGPYETYEGSPVSKGILQYDMWNVTPTDLWDWKVLKEKIAKYGIRN
SLLIAPMPTASTAQILGNNESIEPYTSNIYTRRVLSGEFQIVNPHLLKDLTERGLWHEEMKNQIIACNGSIQSIPEIPDDLKQLYKT
VWEISQKTVLKMAAERGAFIDQSQSLNIHIAEPNYGKLTSMHFYGWKQGLKTGMYYLRTRahhhhhh
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Vector:pNIC-CH2

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 750 mL TB supplemented with 8 g/l glycerol, 50 µg/mL kanamycin and approximately 0.5mL/l 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C. Four hours after inoculation 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 (4,400 x g, 10 min, 4 °C). The resulting cell pellet (17.0 g wet cell weight) was resuspended in lysis buffer (1.5 mL/g cell pellet), supplemented with 500 U Benzonase (Merck) and half a 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, 30,000 NMWL (Millipore) to 27.1 mg/mL in a volume of 0.17 mL.

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 (49,100 x g, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
dATP and magnesium**MassSpec:**
Crystallization: Prior to crystallization, 5mM dATP and 5mM MgCl2, was added to the protein solution that were diluted from 27.1 to 15 mg/mL, using 20 mM HEPES, 300 mM NaCl, 10% glycerol, 2 mM TCEP, pH 7.5. Crystals were obtained by the sitting drop vapour diffusion method in a 96-well 3-drop plate (Corning 3550). 0.1 µl protein solution (15 mg/mL) was mixed with 0.1 µl of well solution consisting of 0.2 M ammonium acetate, 0.1 M bis-tris, pH 5.5 and 25% PEG 3350. The plate was incubated at 20 ºC and crystals appeared in five days. The crystals were transferred to a cryo solution consisting of 0.3 M ammonium acetate, 0.1 M bis-tris, pH 5.5, 29% PEG 3350, 20% glycerol and 0.3 M NaCl, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data to 2.3 Å resolution was collected from a single crystal at ESRF (ID23-1). Crystal belonged to P21 space group with cell parameters of a=66.95 Å, b=129.3 Å, c=76.67 Å, β=95.5. The asymmetric unit contains a dimer.
Data Processing: The Xray data was processed by XDS and scaled by XSCALE. The structure was solved by molecular replacement using MOLREP with yeast R1 protein structure (1ZYZ) as a search model. dATP and Magnesium ions were present in the specificity site. The MR solution was further refined with REFMAC5. Final R-values were R=19.2% and Rfree=25.8%. Coordinates and structure factors are deposited to the protein data bank with accession code 2WGH.