RND1

PDB:2CLS

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:RND1A-s001
Entry Clone Source:MGC
SGC Clone Accession:
Tag:N-terminal hexahistidine tag followed by the TEV proteiease cleavable sequence.
Host:BL-21 (DE3)R3 Rosetta plasmid

Construct

Prelude:sm residues are a result of the construct design.
Sequence:

```
sm RAPQPVVARCKLVLVGDVQCGKTAMLQ VLAKDCYPETYVPTVFENYTACLETEEQR VELSLWDTSGSPYYDNVRPLCYSDSD
AVL LCFDISRPETVDSALKKWRTEILDYCPST RVLLIGCKTDLRTDLSTLMELSHQKQAPI SYEQGCAIAKQLGAEIYLEGSAF
TSEKSI HSIFRTASMLCLNKPSPLPQKSPV
```

Vector:pLIC- SGC1

Growth

Medium:
Antibiotics:
Procedure:2.5µl of construct DNA was added to a well of a PCR plate on ice.
90 µl of competent BL21(DE3)-R3/Rosetta bacteria were added, with the plate remaining on ice. The plate was left on ice for a further 25 minutes. The plate was transferred to a 42°C water bath for 45 seconds and then returned to sit on ice for 2 minutes.
100 µl of SOC medium (pre-warmed to 42°C) was added and the plate incubated at 37°C for 60 minutes.
180 µl of the culture was plated out onto LB-agar containing 50µg/ml kanamycin and 34µg/ml chloramphenicol in a 5.5cm Petri dish. The plate were incubated at 37°C overnight.
30 ml of LB containing 50µg/ml Kanamycin and 34µg/ml chloramphenicol was prepared in a 250 ml flasks were prepared. Two colonies, from the transformation plate, were added to each starter culture. The flask was left in a shaker at 200 rpm and 37°C overnight.
19 ml of the starter culture was added to autoclaved 1litre TB medium that contained 50µg/ml kanamycin 34µg/ml chloramphenicol . The flasks were placed in a shaker at 180 rpm and 37°C. When the cell density reached an OD600 nm of approximately 1.0 the temperature was reduced to 20°C for 1 hr before induction with the addition of 1 mM IPTG.
The cells were spun down (4000 rpm, 10 mins). The pellets were resuspended in 30 ml lysis/ binding buffer, transfered into 50 ml tubes and placed in a -80°C freezer.
Lysis buffer: 50 mM HEPES pH 7.5; 10 mM Imidazole pH 7.5; 100 mM NaCl, 2 mM MgCl 2

Purification

Procedure
Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)
The cell extract was loaded on the column at 0.8 ml/min on an AKTA-express system (GE/Amersham). The column was then washed with 10 column volumes of Affinity Binding buffer, 10 column volumes of Affinity wash buffer, the 10 column volumes of Affinity Wash Buffer II and RND1A eluted with Affinity elution buffer at 0.8 ml/min. The eluted peak of A280 was automatically collected.

Column 2 : Gel filtration, Hiload 16/60, S75 16/60 - 120 ml
The eluted fractions from the Ni-affinity Histrap columns were loaded on the gel filtration column in GF buffer at 1.0 ml/min. Eluted proteins were collected in 1 ml fractions.

Enzymatic treatment: At this stage the purity of the protein was greater than 95 % based on SDS - PAGE analysis. The C-terminal hexahistidine tag was removed by TEV protease treatment. The TEV protease, a hexahistidine-tagged construct, was over-expressed and purified in-house to a final concentration of 2.5 mg/ml.
Add 30µl of the TEV protease was added to each fraction and left at 4°C overnight. The following steps were carried out to remove the cleaved products and TEV protease.
Place 200µl of 50 % Ni-NTA agarose in a 1.5 ml eppendorf tubes, add 1ml of 50 mM Tris pH 8, 150 mM NaCl mix, spin down and remove buffer. Repeat this resin wash step once.
Add the TEV treated protein sample to the resin and mix for 30 min. Finally spin down resin and collect the supernatant which contains the cleaved RND1A.
Before the final concentration step, the protein concentration was measured and 5 molar equivalents of GTP was added. The sample was then concentrated to 36 mg/ml using a 10 kD cutoff spin concentrator.

Extraction

Procedure
After thawing, the resuspended cells were lysed by passing through the Emusiflex C5 high pressure homogeniser. PEI (stock 5 %) was added to the homogenate to a final concentration of 0.15%. The cell debris, nuclei and DNA were spun down at 16,500 rpm for 45 min. The supernatant was collected and 0.5 mM GTP added.
Concentration:
Ligand
MassSpec: Before His-Tag removal: expected MWt: 24551; After His-Tag removal: expected MWt: 22085, measured MWt: 22084.8.
Crystallization: Initial crystal hits produced small crystals that were crushed and used for seeding of a screen around the initial reservoir conditions. Large single crystals grew from a 2:1 ratio mix of RND1(Mg 2+ GTP)-to-reservoir (0.2M NaI, 0.1 M BT Prop pH 8.5, 20 % PEG 3350 , 10 % ethylene glycol, 0.5 % DMSO).
NMR Spectroscopy:
Data Collection: Resolution: 2.3Å; X-ray source: Synchrotron SLS-X10, single wavelength.
Data Processing: