RAC3A

PDB:2C2H

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:RAC 3A-s001
Entry Clone Source:MGC
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with a TEV cleavage site
Host:BL-21(DE3)R3

Construct

Prelude: This is a mutant form of RAC 3 with the Cys178 replace by Gly. The Cys178Gly mutation is indicated by *
Sequence:

SMQAIKCVVVGDGAVGKTCLLISYTTNAF PGEYIPTVFDNYSANVMVDGKPVNLGLWD TAGQEDYDRLRPLSYPQTDVFLICFSL VS PASFENVRAKWYPEVRHHCPHTPILLVGT KLDLRDDKDTIERLRDKKLAPITYPQGLA MAREIGSVKYLECSALTQRGLKTV FDEAI RAVLG*

Vector:pNIC28-Bsa4

Growth

Medium:
Antibiotics:
Procedure:

Purification

Procedure
Column 1 : Low pressure chromatography using Bio-Rad Econo column (2.5 cm x 13 cm).
Total volume of Ni-NTA added to BioRad drip column: 4 mls (50%).
Resin washed with 12.5 ml of WB1. The supernatant was applied to a column using 5 ml pipette and allowed to pass over the resin. The flow through was collected in a 50 ml falcon tube and applied once more to the column. Two wash steps followed. Wash with 12.5 ml of WBI. Wash with 12.5 ml column vols of WBII. Elute with 14 mls of EB into 7x2 ml fractions.
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.

Enzymatic treatment : Add 30 microl 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.Change buffer from Elution Buffer to 50 mM Tris pH 8, 150 mM NaCl, 10 mM MgCl 2 using a 10-kD cutoff concentrator.
Place 200 ml 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 RAC 3A.

Extraction

Procedure
All extraction steps were carried out at 4 degC.
1 tablet protein inhibitor in 10ml EX buffer was added to the 1L growth pellet. Total vol: 45 mls (estimate). Cell breakage: 5 passes through the Emulsiflex C5 high pressure homogeniser. Total vol: 50 mls (estimate). Centrifuge for 40 mins at 16000 rpm and 4°C to remove cell debris. Discard pellet.
Concentration: The concentration of RAC3A was measured and 5 molar equivalents of GDP were added before concentrating to a 15 mg/ml. The conentrated protein was aliquoted into 50 µl volumes before freezing in the -80°C freezer.
Ligand
MassSpec: Before His-tag removal: Expected MWt: 22320.3. Measured MWt: 22319.9.

After His-tag removal: Expected MWt: 19854.7. Measured MWt: 19854.3.
Crystallization: Crystals grew from a 2:1 ratio mix of RAC 3A(Mg 2+ GDP)-to-reservoir (0.5 M ammonium sulphate, 0.1 M Hepes pH 7.5, 30 % MPD).
NMR Spectroscopy:
Data Collection: Resolution: 1.9 Å; X-ray source: Synchrotron SLS -X10, single wavelength.
Data Processing: