RGS6

PDB:2ES0

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:RGS6A-s001
Entry Clone Source: Origene
SGC Clone Accession:
Tag:N-terminal, TEV cleavable hexahistidine tag.
Host:BL-21(DE3)R3 phage resistant strain

Construct

Prelude:
Sequence:

Vector:pNIC28-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Freshly transformed E. coli cells was used to inoculate 1 litre of TB plus 50 µg/ml kanamycin. When OD600 reached ~2.0 the temperature was shifted down from 37°C to 25°C for 1 hour before induction with the addition of 1 mM IPTG. Protein expression was allowed to carry on for a further 4 hours before harvest.

Purification

Procedure
Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)

Buffers: Binding Buffer: 50 mM Hepes pH 8.0, 500 mM NaCl, 5 % Glycerol, 5 mM Imidazole pH 8.0, 0.5 mM TCEP. Wash Buffer: 50 mM Tris pH 8.0, 500 mM NaCl, 5 % Glycerol, 25 mM Imidazole pH 8.0, 0.5 mM TCEP. Elute Buffer: 50 mM Hepes pH 8.0, 500 mM NaCl, 5 % Glycerol, 250 mM Imidazole pH 8.0, 0.5 mM TCEP.

Procedure: 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 Binding Buffer, 10 column volumes of Wash Buffer, and then eluted with Elute 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.

Buffers : GF Buffer: 50 mM Tris pH 8.5, 500 mM NaCl, 0.5 mM TCEP.

Procedure: 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. Using a Centricon 5 K cutoff concentrator, an attempt was made to concentrate the RGS6A-p024 pooled fractions. However, at this stage the protein failed to concentrate and began to precipitate. The hexahistidine tag was cleaved before buffer exchange which was the procedure that allowed the protein to be concentrated.

Enzymatic treatment : Add 30 µl 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 MgCl2 using a 10-kD cutoff concentrator. Place 200 m l 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 RGS6A.

Buffer exchange: Exchange Buffer: 10mM Na citrate, pH 5.5, 500mM NaCl, 0.5mM TCEP.

Procedure: Concentration in GF buffer resulted in the protein precipitation. After centrifugation (12,000 x g, 5 min, 4°C) the protein concentration of the supernatant was determined as 2.2 mg/ml, from the absorbance at 280 nm using a molar extinction coefficient of 19,680 M-1*cm-1. The protein was subjected to a screen for buffer stability, using a fluorescence-based thermal stability shift assay (using the dye SYPRO orange, Molecular Probes). Tested buffers were at 100 mM concentration and the protein concentration was 0.075 µg/ml. Analysis of the data suggested acidic pH conditions in combination with high NaCl salt as being beneficial in terms of protein stability. Consequently, the remaining supernatant was diluted into 3-fold volume of 10 mM Na citrate, pH 5.5, 500 mM NaCl, 0.5mM TCEP. The sample then could be concentrated (VivaSpin 4, 5 MWCO) to 30 mg/ml. The sample was immediately set up for crystallization.

Characterization of quaternary structure in Exchange buffer: Analytical ultracentrifugation velocity analysis (XL-A, Beckman-Coulter, 45,000rpm, protein concentration: 0.5 mg/ml) was performed to analyse the quaternary structure of the sample. Two peaks observed corresponded to the presence of monomer and dimer species, with the dimer peak comprising approx. 40 % of the total protein.

Extraction

Procedure
Resuspension buffer (RS): 50 mM Tris pH 8.0, 500 mM NaCl, 5 % Glycerol, 5 mM Imidazole pH 8.0, 0.5 mM TCEP. 1 proteinase inhibitor tablet (EDTA free) was added to 10ml Resuspension Buffer. This was used to suspend the pellet before homogenisation. 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:
Ligand
MassSpec:
Crystallization: Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)

Buffers: Binding Buffer: 50 mM Hepes pH 8.0, 500 mM NaCl, 5 % Glycerol, 5 mM Imidazole pH 8.0, 0.5 mM TCEP. Wash Buffer: 50 mM Tris pH 8.0, 500 mM NaCl, 5 % Glycerol, 25 mM Imidazole pH 8.0, 0.5 mM TCEP. Elute Buffer: 50 mM Hepes pH 8.0, 500 mM NaCl, 5 % Glycerol, 250 mM Imidazole pH 8.0, 0.5 mM TCEP.

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NMR Spectroscopy:

Data Collection:

Data Processing: