RGS2 Gαi3 complex

Entry clone source: MGC

Entry clone accession : RGS2A-s001

Construct: Prof D Siderovski

Tags and additions: N-terminal hexahistidine tag

Host : BL-21(DE3)R3 (phage resistant)

Sequence:
MSSGVDLGTENLYFQSNAKPSPEEAQLWS
EAFDELLASKYGLAAFRAFLKSEFSEENI
EFWLACEDFKKTKSPQKLSSKARKIYTDF
IEKEAPKEINIDFQTKTLIAQNIQEATSG
CFTTAQKRVYSLMENDSYPRFLKSEFYQD
LCKKPQITTEPH
RGS2 mutations
Cys106-to-serine (C106S), Asn184-to-aspartate (N184D), and Glu191-to-lysine (E191K)

Growth medium, induction protocol:
Tranformation: 2 µl of the RGS2A mutant construct was added and mixed to 100 µl of BL21(DE3)-Phage Resistant competent cells in a sterile 96-well microtitre plate on ice. The plate was left on ice for a further 10 minutes. The heat-shock procedure was done by transferring the plate to a 42°C water bath for 45 seconds and then returning it to ice for a further 2 minutes. 200 µl of LB medium was added to the well and the plate incubated at 37°C for 40 minutes. The entire 150 µl culture was plated out onto LB-Amp agar in a 5.5 cm Petri dish. The plates were incubated at 37°C overnight.
Large scale expression: The RGS2A mutant fresh transformants were used to innoculate 30 ml TB media with 100 µg/ml Ampicillin which was placed in a 37°C shaker overnight. The next day this starter culture was used to innoculate 6 x 1 litre of TB medium which contained 100 µg/ml Ampicillin. Protein induction was carried out with the addition of 0.5 mM IPTG after the cells reached an OD_{600} of 0.7 and the incubation temperature decreased to 20°C. After 14 hours the cells were harvested by centrifugation. The cell pellet was frozen in the -80°C freezer.
Lysis Buffer: 50 mM Hepes pH 7.5, 300 mM NaCl, 5 % Glycerol, 10 mM Imidazole

Extraction buffer, extraction method : 1 tablet protein inhibitor in 10 ml Lysis Buffer was added to the 1L growth pellet.
Total vol: 45 mls (estimate).
Cell breakage: 3 passes through the Emulsiflex C5 high pressure homogeniser.
Total vol: 50 mls (estimate).
Centrifuge for 45 mins at 16000 rpm and 4°C to remove cell debris. Discard pellet.

Column 1: Low pressure chromatography using Bio-Rad Econo column (2.5 cm x 13 cm).

Buffers: Wash Buffer I (WBI): 50 mM Hepes pH 7.5, 300 mM NaCl, 5 % glycerol, 10 mM imidazole; **Wash Buffer II** (WBII): 50 mM HEPES pH 7.5, 300 mM NaCl, 5 % glycerol, 30 mM imidazole; **Elute Buffer** (EB): 50 mM Hepes pH 7.5, 300 mM NaCl, 5 % glycerol, 250 mM imidazole

Procedure: Total volume of Ni-NTA added to BioRad drip column: 4 ml (50 %). Resin washed with 12.5 ml of WBI. The supernatent 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 ml of EB into 6x2 ml fractions.

Column 2 : Size exclusion using a S200 16/60 column
Buffers : GF Buffer: 50 HEPES pH 8.0, 150 mM NaCl, 5% glycerol, 0.5mM TCEP
Procedure: The column was pre-equilibrated with two column volumes of GF buffer (flow rate 1 ml/min). The fractions from gel filtration that contained RGS2A were pooled and concentrated before loading on to S200 16/60 gel filtration column. The fractions containing protein were identified on a coomasie blue stained gel.
Enzymatic treatment : His-Tag removal: Each Gel filtration fraction containing RGS 1 were cleaved overnight with 20 µl of 2.8 mg/ml Tev protease. Ni-NTA beads (Qiagen) were added to the protein/Tev protease mixture to remove the His tag with Tev and other contaminants. The protein was concentrated to a final concentration of 400 µM
Mass spec characterization : The expected mass of RGS2A mutant without histidine tag is 16439.5. The experimentally determined mass was 16439.5.

G protein αi3

Entry Clone Source: Prof D Siderovski
Vector: pProEXHT
Sequence: MREVKLLLLGAGESGKSTIVKQMKIIHEA GYSEEECKQYKAVVYSNTIQSIIAIIRAM GRLKIDFGDSARADDARQLFVLAGAAEEG FMTAELAGVIKRLWKDSGVQACFNRSREY QLNDSAAYYLNDLDRIAQPNYIPTQQDVL RTRVKTTGIVETHFTFKDLHFKMFDVGGQ RSERKKWIHCFEGVTAIIFCVALSDYDLV LAEDEEMNRMHESMKLFDSICNNKWFTDT SIILFLNKKDLFEEKIKKSPLTICYPEYA GSNTYEEAAAYIQCQFEDLNKRKDTKEIY THFTCATDTKNVQFVFDAVTDVIIKNNLK DCGLF
Growth medium, induction protocol: **Tranformation:** 2µl of the construct was added and mixed to 90µl of -phage-resistant BL21 (DE3) with the rosetta plasmid and left on ice for 20 minutes. The heat-shock procedure was done by transferring the plate to a 42°C water bath for 45 seconds and then returning it to ice for a further 2 minutes. 100µl of LB medium (pre-warmed to 42°C) was added to the well and the plate incubated at 37°C for 40 minutes. **Large scale expression:** The Galpha3i fresh transformants were used to innoculate 20 ml LB media with 100 µg/ml Ampicillin which was placed in a 37°C shaker overnight. The next day this starter culture was used to innoculate 4 x 1 litre of TB medium which contained 100µg/ml Ampicillin. Protein induction was carried out with the addition of 0.5 mM IPTG after the cells reached an OD_{600} of 0.7 and the incubation temperature decreased to 20°C. After 16 hours the cells were harvested by centrifugation. The cell pellet was frozen in the -80°C freezer.
Extraction Method and Extraction buffer: Lysis Buffer: 50 mM Hepes pH 7.5, 300 mM NaCl, 5 % Glycerol, 10 mM imidazole. 1 tablet protein inhibitor in 10 ml Lysis Buffer was added to the 1L growth pellet. **Total vol:** 45 mls (estimate). **Cell breakage:** 3 passes through the Emulsiflex C5 high pressure homogeniser. Centrifuge for 45 mins at 16000 rpm and 4 C to remove cell debris.
Column 1 : Low pressure chromatography using Bio-Rad Econo column (2.5 cm x 13 cm).

Buffers: Wash Buffer I (WBI): 50 mM Hepes pH 8.0, 300 mM NaCl, 5 % glycerol, 10 mM imidazole; **Wash Buffer II:** 50 mM Tris pH 8.0, 300 mM NaCl, 5 % glycerol , 30 mM imidazole; **Elute Buffer:** 50 mM Hepes pH 8.0, 300 mM NaCl, 5 % glycerol, 250 mM imidazole.
Procedure: Total volume of Ni-NTA added to BioRad drip column: 4 ml (50 %). Resin washed with 12.5 ml of WBI. 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 ml of EB into 6x2 ml fractions.
Column 2: Gel filtration
Procedure: The fractions from gel filtration that contained G protein a1 were pooled and concentrated before loading on to S200 16/60 gel filtration column. The fractions containing protein were identified on a coomasie blue stained gel.
Enzymatic treatment: His-Tag removal. The eluate was treated with Tev protease. The sample was rebound to Ni sepharose to remove Tev and other contaminants. The His tag cleaved protein was concentrated to 300 µM.
Mass spec characterisation: The expected mass of N terminally 30 aminoacids truncated GNAI3A and experimentally determined mass was 40313**.**

Common steps

Purification of G protein αi3 and RGS2A complex: Purified G protein and RGS2 mutant was mixed in the ratio 1:1.5 and incubated at 4°C for 20 minutes. The sample was passed through S200 gel filtration column which was pre-equilibrated with 25 mM Hepes pH 7.5, 150 mM NaCl, 5 % glycerol, 2 mM DTT, 100 µM AlCl3, 20 mM NaF and 100 µM GDP. The proteins eluted as a complex was analysed using PAGE and the fractions were pooled and concentrated to 23 mg/ml and used for crystallisation set up.
Crystallisation: Crystals grew from a 2:1 ratio mix of G proteinαi3 , RGS2A and precipitant containing 0.1 M Hepes pH 7.5 and 2 M ammonium sulphate.
Data acquisition and analysis: Resolution: 2.8Å; **X-ray source:** Rigaku/MSC FR-E rotating anode x-ray generator.