Entry Clone Source: MGC

Entry Clone Accession: (IMAGE:4178593)

SGC Construct ID: VIPR2A-c104

GenBank GI number: gi|21361557

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Coding DNA sequence:

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CATATGCACCATCATCATCATCATTCTTCT
GGTGTAGATCTGGGTACCGAGAACCTGTAC
TTCCAATCCATGCGATTTCATCTGGAAATA
CAGGAGGAAGAAACAAAATGTGCAGAGCTT
CTGAGGTCTCAAACAGAAAAACACAAAGCC
TGCAGTGGCGTCTGGGACAACATCACGTGC
TGGCGGCCTGCCAATGTGGGAGAGACCGTC
ACGGTGCCCTGCCCAAAAGTCTTCAGCAAT
TTTTACAGCAAAGCAGGAAACATAAGCAAA
AACTGTACGAGTGACGGATGGTCAGAGACG
TTCCCAGATTTCGTCGATGCCTGTGGCTAC
AGCGACCCGGAGGATGAGAGCTGACAGTAA
AGGTGGATACGGATCCGAA
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Tags and additions: Cleavable N-terminal His6 tag (* TEV cleave site).

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain)

Final protein sequence (small letters refer to tag sequence):

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mhhhhhhssgvdltenlyfq*SMRFHLEIQ
EEETKCAELLRSQTEKHKACSGVWDNITCW
RPANVGETVTVPCPKVFSNFYSKAGNISKN
CTSDGWSETFPDFVDACGYSDPEDES
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Growth medium, induction protocol: 5 ml from an overnight culture containing 50 µg/ml kanamycin & 34 µg/ml chloramphenicol was used to inoculate each 1 L culture of LB. Cultures were grown at 37 °C until the OD_{600} reached ~1.8 then the temperature was adjusted to 20 °C. Expression was induced using 0.5 mM IPTG added at an OD_{600} of ~2.0 and the cells were grown overnight. The cells were collected by centrifugation, and the pellet was resuspended in binding buffer and frozen. For selenomethionine labeling of protein, 50 ml of an overnight culture was washed with methionine minus medium (Molecular Dimensions Ltd.) and used to inoculate 8 L of methionine minus medium supplemented with 40 mg / L of L-selenomethionine (Acros Organics) containing 50 µg/ml kanamycin & 34 µg/ml chloramphenicol. Protein expression was induced as above for the native protein.

Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl.

Extraction buffer, extraction method: Frozen pellets were thawed and cells lysed with 3 passes through a C3 high pressure cell disrupter (Avestin). The lysate was centrifuged at 17,000 rpm for 30 minutes and the pellet was retained for purification. The pellet was solubilized in solubilization buffer using a Teflon homogenizer followed by mixing on a rotating platform for 1 hour at room temperature. The solubilized lysate was centrifuged at 17,000 rpm for 30 minutes and the supernatant collected for purification.

Column 1: Ni-affinity. Nickel-IDA (Sterogene), 5 ml of 50 % slurry in 1.5 x 10 cm column, washed with solubilization buffer.

Buffers:

Solubilization buffer: 25 mM HEPES, pH 7.5; 8 M Urea; 1 M NaCl; 5 mM imidazole and 0.5 mM TCEP

Wash buffer: 25 mM HEPES, pH 7.5; 8 M Urea; 1 M NaCl; 30 mM imidazole and 0.5 mM

TCEP
Elution buffer: 25 mM HEPES, pH 7.5; 8 M Urea; 1 M NaCl; 50 to 400 mM imidazole and 0.5 mM TCEP

Procedure: The solubilized lysate was loaded by gravity flow on the Ni-IDA column. The column was then washed with 100 ml solubilization buffer and 100 ml of wash buffer. The protein was eluted by applying 10 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 150, 250 and 400 mM); fractions were collected until essentially all protein was eluted.

Protein Refolding: Eluted protein was transferred to dialysis tubing (3 kDa cut-off) and dialysed against 2 L of refolding buffer overnight at 4°C, followed by a further 8 hour dialysis in fresh refolding buffer. The protein was then dialysed overnight against 1 L of dialysis buffer overnight at 4°C.

Buffers:
Refolding buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol, 0.4 M arginine, 0.2 mM reduced glutathione, 0.2 mM oxidised glutathione and 5 mM EDTA
Dialysis Buffer: The protein was then dialysed overnight against 50 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol

Column 2: Size Exclusion Chromatography. Superdex S75 16/60 HiLoad

Buffers: 50 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol

Procedure: The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated in 50 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol using an ÄKTAxpress system.

Mass spectrometry characterization: LC-ESI MS TOF analysis indicated the purified protein had a mass of 13241.7 Da. The measured mass is in accordance with the expected native mass (13154 Da) with the incorporation of two selenomethionines and the loss of 6 Da as a result of formation of 3 disulphide bonds.

Protein concentration: Protein was concentrated to 25 mg/ml using a 3 kDa cut-off concentrator (Amicon).

Crystallization: Crystals were grown at 20 °C in 150 nl sitting drops comprising a 1:1 ratio of protein (25mg/ml) to reservoir solution containing 40% PEG 300; 0.1 M citrate/PO4 pH 4.2

Data Collection: Crystals were vitrified directly in liquid nitrogen. "Native" and SAD datasets were collected from crystals grown with selenomethionine-substituted protein on beamline I03 at the Diamond Light Source. Phasing information was obtained from a 2.9 Å Se-SAD dataset collected at the selenomethionine peak wavelength (λ = 0.9799 Å). High resolution "native" data, extending to 2.1 Å resolution, were obtained from similarly substituted crystals collected at a wavelength below the peak (λ = 0.9809 Å).