Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4858949

SGC Construct ID: CDC25CA-c005

GenBank GI number: gi|4502707

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

Amplified construct sequence:

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CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGACTCAG
ATGCTGGAGGAAGATTCTAACCAGGG
GCACCTGATTGGTGATTTTTCCAAGG
TATGTGCGCTGCCAACCGTGTCAGGG
AAACACCAAGATCTGAAGTATGTCAA
CCCAGAAACAGTGGCTGCCTTACTGT
CGGGGAAGTTCCAGGGTCTGATTGAG
AAGTTTTATGTCATTGATTGTCGCTA
TCCATATGAGTATCTGGGAGGACACA
TCCAGGGAGCCTTAAACTTATATAGT
CAGGAAGAACTGTTTAACTTCTTTCT
GAAGAAGCCCATCGTCCCTTTGGACA
CCCAGAAGAGAATAATCATCGTGTTC
CACTGTGAATTCTCCTCAGAGAGGGG
CCCCCGAATGTGCCGCTGTCTGCGTG
AAGAGGACAGGTCTCTGAACCAGTAT
CCTGCATTGTACTACCCAGAGCTATA
TATCCTTAAAGGCGGCTACAGAGACT
TCTTTCCAGAATATATGGAACTGTGT
GAACCACAGAGCTACTGCCCTATGCA
TCATCAGGACCACAAGACTGAGTTGC
TGAGGTGTCGAAGCCAGAGCAAAGTG
CAGGAAGGGGAGCGGCAGCTGCGGGA
GTAAGACAGTAAAGGTGGATACGGAT
CCGAA
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Final protein sequence (Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^SMTQ
MLEEDSNQGHLIGDFSKVCALPTVSG
KHQDLKYVNPETVAALLSGKFQGLIE
KFYVIDCRYPYEYLGGHIQGALNLYS
QEELFNFFLKKPIVPLDTQKRIIIVF
HCEFSSERGPRMCRCLREEDRSLNQY
PALYYPELYILKGGYRDFFPEYMELC
EPQSYCPMHHQDHKTELLRCRSQSKV
QEGERQLRE
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^ TEV cleavage site

Tags and additions: Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 50µl of LB culture started from glycerol stocks was added to 50ml fresh Minimal Pink Medium (MCSG) containing 1.5mg kanamycin, 7.5mg amplicillin, 0.05mg vitamin B1, and 0.135mg vitamin B12, M9 salts, non-inhibitory amino acids, metal supplements, glucose and glycerol as described in paper (Donnelly, MI *et al*, 2004). Cultures were grown overnight at 37°C (150rpm). The 50ml overnight culture was divided equally into two 1L of freshly prepared Minimal Pink Medium. Cultures were grown at 37°C (180rpm) until the OD_{600} reached ~1.0. Next, protein expression was induced using 0.5 mM IPTG. Cultures were switched to an 18°C re-suspended in lysis buffer and frozen in -80°C.
Lysis buffer: 50 mM HEPES, pH 8.0; 500 mM NaCl; 20 mM Imidazole; 5% Glycerol 10 mM β-mercaptoethanol.
Extraction buffer, extraction method: Frozen cell pellets were thawed and fresh lysozyme was added at a final concentration of 1mg/ml to the 40mL of lysate. Cells were further lysed using sonication (Misonix 3000). The lysate was centrifuged (RC5C-Plus centrifuge, Sorval SS-34 rotor) at 17,500rpm for 80 minutes and the supernatant was filtered through a 0.45µm in line filter (Pall) prior to loading on nickel columns (GE HS) using ÄKTA Xpress.

Column 1: Immobilized metal affinity chromatography I (IMAC I) using ÄKTA Xpress (GE HS).

Column 1 Buffers:
Desalting buffer: 50 mM HEPES, pH 8.0; 500 mM NaCl; 5% glycerol; 10 mM β-mercaptoethanol.
Lysis buffer: 50 mM HEPES, pH 8.0; 500 mM NaCl; 5% glycerol; 20 mM Imidazole; 10 mM β-mercaptoethanol.
Elution buffer: 50 mM HEPES, pH 8.0, 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 10 mM β-mercaptoethanol.

Column 1 Procedure: IMAC I using a 5ml HiTrap Chelating HP column charged with Ni^{+2} ions and buffer exchange chromatography on a HiPrep 26/10 desalting column (both GE HS) were performed using ÄKTA Xpress (GE HS). The His_6 tag was cleaved using the recombinant TEV protease expressed from the vectore pRK508[4] (a gift from Dr. D. Waugh, NCI). The TEV protease was added to the target protein in a ratio of 1:50 and the solution was incubated at 4°C for 48 hours.

Column 2: Immobilized metal affinity chromatography II (IMAC II) using ÄKTA Xpress (GE HS).

Column 2 Buffers:
Lysis buffer: 50 mM HEPES, pH 8.0; 500 mM NaCl; 5% glycerol; 20 mM Imidazole; 10 mM β-mercaptoethanol.
Elution buffer: 50 mM HEPES, pH 8.0; 500 mM NaCl; 5% glycerol; 250 mM Imidazole; 10 mM β-mercaptoethanol.

Column 2 Procedure: The proteins with His_6 tag removed were purified IMAC II using a 5ml HiTrap Chelating HP column (GE HS) charged with Ni^{+2} ions. Protein was eluted and collected at Imidazole concentrations of 20 mM and 35 mM.

Reductive Methylation Procedure: 10-20mg of purified protein at a concentration of 5-10mg/ml was prepared. 40µg of 1 M formaldehyde per 1mL of protein solution was added. Immediately after, 20µl of 1 M ABC per 1mL of protein solution was added and gently mixed. The solution was incubated at 4°C for 2 hours and the addition of formaldehyde and ABC was

repeated. At the end of the 2nd incubation, an additional 10µl of ABC was added per 1mL of protein solution. The solution was incubated at 4°C overnight (12-14 hours). The following day, 5mg of glycine (using 5mg/mL stock) and 5 mM DTT were added to quench the reaction and the solution was left on ice for 2 hours.

Mass spectrometry characterization: Not determined.

Protein concentration: Protein was buffer exchanged several times in crystallization buffer (20 mM HEPES pH 8.0, 250 mM NaCl, and 2 mM dithiothreitol (DTT)) during concentration and concentrated to 43.03mg/ml using an Amicon Ultra 15 - 3kDa cut-off concentrator.

Crystallisation: Crystals grown at 16°C in hanging drops from a 1:1 ratio of reservoir solution (23% PEG 3350, 1 M Bis-Tris pH 5.5, 2 M ammonium sulfate) and methylated protein (25mg/ml).

Data collection: Crystals were frozen in the presence of 10% glycerol (23% PEG 3350, 1 M Bis-Tris pH 5.5, 2 M ammonium sulfate, 10% glycerol) before used for the data collection. Rod-shape crystals (0.2x0.05x0.05) diffracted to 2.6Å. The data were collected on ADSC Q315r, at the wavelength of 0.97929Å, 5 second exposures during a one degree rotation per diffraction frame.
X-ray source: Data were collected with Synchrotron radation at the structural biology center (SBC) at Advanced Photon Source (APS) at Argonne, IL.
Phasing: The structure was determined by molecular replacement using balbes on ccp4 using the structure of CDC25B (PDB: 2IFV) as the search model.