BIN2A (4AVM) Materials & Methods

Entry clone source: MGC (IMAGE)

Entry clone accession: IMAGE:5722815

SGC Construct ID: BIN2A-c011

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

DNA sequence:

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ATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACC
TGTACTTCCAATCCATGGGCCTCTTC
GCCAAGCAGGTGCAGAAGAAGTTTAG
CAGGGCCCAGGAGAAGGTGCTGCAGA
AATTGGGGAAAGCTGTAGAAACCAAA
GATGAACGATTTGAACAAAGCGCTAG
CAACTTCTACCAACAACAGGCAGAAG
GCCACAAGCTGTACAAGGACCTGAAG
AACTTCCTTAGTGCAGTCAAAGTGAT
GCATGAAAGTTCAAAAAGAGTGTCAG
AAACCCTGCAGGAGATCTACAGCAGC
GAGTGGGACGGTCATGAGGAGCTGAA
GGCCATCGTATGGAATAATGATCTCC
TTTGGGAAGACTACGAGGAGAAACTG
GCTGACCAGGCTGTAAGGACCATGGA
AATCTATGTTGCCCAGTTCAGTGAAA
TTAAGGAGAGAATTGCCAAGCGGGGT
CGGAAACTCGTGGACTATGACAGTGC
CCGACACCACCTGGAGGCAGTGCAGA
ATGCCAAGAAGAAAGATGAGGCCAAG
ACTGCCAAGGCAGAGGAAGAGTTCAA
CAAAGCCCAGACTGTGTTTGAAGATC
TGAACCAAGAACTACTAGAGGAGCTG
CCTATTCTTTATAATAGTCGTATTGG
CTGCTATGTGACCATCTTCCAAAACA
TTTCCAACTTGAGGGATGTCTTCTAC
AGGGAAATGAGCAAGCTGAACCACAA
TCTCTACGAGGTGATGAGCAAACTGG
AGAAGCAACATTCCAATAAATGA
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Final protein sequence (His_6 affinity Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smGL
FAKQVQKKFSRAQEKVLQKLGKAVET
KDERFEQSASNFYQQQAEGHKLYKDL
KNFLSAVKVMHESSKRVSETLQEIYS
SEWDGHEELKAIVWNNDLLWEDYEEK
LADQAVRTMEIYVAQFSEIKERIAKR
GRKLVDYDSARHHLEAVQNAKKKDEA
KTAKAEEEFNKAQTVFEDLNQELLEE
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LPILYNSRIGCYVTIFQNISNLRDVF
YREMSKLNHNLYEVMSKLEKQHSNK

^ TEV protease recognition site
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Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2. (a phage-resistant derivative of Rosetta2 [Novagen]).

Cell Growth and Induction: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1-ml culture in LB (+ 50 µg/ml kanamycin, 35 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots. A loop full of cells from the glycerol stock was inoculated into 60-ml of LB medium containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol and grown overnight at 37°C. Four cultures of 1-L each of TB medium (+ 50 µg/ml kanamycin) in 2.5L UltraYield baffled flasks were inoculated with 15 ml each of the overnight culture. The cultures were grown at 37°C for 4 hours until OD600 of 1. The cultures were then transferred to 19°C, and after 45 minutes IPTG was added to 0.75 mM. Growth was continued overnight. The cells were collected by centrifugation, the pellets were resuspended in 105 ml of Lysis Buffer with the addition of a 1:5000 dilution of Calbiochem Protease inhibitor set VII. The resuspended cell pellet was placed in a -80°C freezer.

Cell Lysis: The re-suspended cell pellet was lysed by sonication. PEI (polyethyleneimine) was added to a final concentration of 0.15 % from a 5% (w/v, pH 7.5) stock, stirring for 15 minutes, then centrifugation for 45 minutes at 25,000 xg.
Lysis buffer: 40mM Tris, pH 8, 250 mM NaCl, 30 mM imidazole, 1 mM TCEP, 10% glycerol

Column 1: Histrap FF 5 ml (GE Healthcare)

Column 1 Buffers:
Affinity buffer: 25 mM Tris, pH 8.0; 250 mM NaCl; 10% glycerol; 0.5 mM TCEP; 30 mM Imidazole.
Elution buffer: 25 mM Tris, pH 8.0; 250 mM NaCl; 10% glycerol; 0.5 mM TCEP; 300 mM Imidazole.

Column 1 Procedure: The cell extract was loaded onto the column at 5ml/min on an ÄKTA-express system (GE Healthcare). The column was then washed with 10 volumes of Affinity buffer, 10 volumes of wash buffer, then eluted with elution buffer at 4ml/min. The eluted peak of A280 was automatically collected.

Enzymatic treatment: The eluted protein was treated with TEV protease (His6-tagged) overnight at 4°C, while being dialysed in Affinity buffer, using 3000 MWCO dialysis tubing. The protease and other impurities were removed by passing through a 3mL bed of Ni-NTA (Generon)

Column 2: Gel filtration, HiLoad 16/60 Superdex 200, 120ml.

Column 2 Buffers:
Gel Filtration buffer: 25 mM Tris, pH 8, 250 mM NaCl, 10% glycerol, 1 mM TCEP

Column 2 Procedure: The protein was loaded and fractionated on the gel filtration column in GF buffer at 1.2ml/min. 2ml fractions were collected at the A280 peaks. The fractions were analyzed by SDS-PAGE and relevant fractions were pooled. This column resulted in a unique peak of the BIN2 protein, at an elution volume compatible with a dimer.

Concentration: The protein was concentrated to 10 mg/ml (concentrations estimated by A280, using an extinction coefficient of 31400 and molecular mass of 27922.6 Da).

Crystallization: Crystals were grown by vapor diffusion at 20°C in 150nl sitting drops. A crystal was grown by mixing 75nl of protein solution (10mg/ml) and 75nl of precipitant consisting of 30% Jeffamine 2001, 100mM NaI, 0.1M HEPES pH 7.0. Crystals appeared in about a week at 20°C. Crystals were cryo-protected by bathing the crystal in mother liquor supplemented with 25% Ethylene glycol and flash-freezing in liquid nitrogen.

Data Collection: Diffraction data for the native dataset were collected from a single crystal at the Diamond synchrotron beamline I04, at a wavelength of 0.9795Å. The N-BAR domain of human Amphyphysin (PDB code 3SOG) was used as a search model for molecular replacement using PHASER MR, part of the PHENIX suite. The structure was refined to an Rwork/RFree of 0.1637 / 0.2028 using autoBUSTER. Deposited as PDB code 4AVM.