PIP4K2A

PDB:2YBX

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:gi:17390057
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:PIP4K2AA-k004
Tag:N-terminal cleavable hexahistidine tag : MHHHHHHSSGVDLGTENLYFQSM
Host:E.coli BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqs*mDPLLSVLMWGVNHSINELSHVQIPVMLMPDDFKAYSK IKVDNHLFNKENMPSHFKFKEYCPM
VFRNLRERFGIDDQDFQNSLTRSAPLPNDSQARSG ARFHTSYDKRYIIKTITSEDVAEMHNILKKYHQYIVECHGITLLPQFLGMY
RLNVDGVEI YVIVTRNVFSHRLSVYRKYDLKGSTVAREASDKEKAKELPTLKDNDFINEGQKIYIDDNN KKVFLEKLKKDVEFLA
QLKLMDYSLLVGIHDVERAEQEEVECEENDGEEEGESDGTHPVG TPPDSPGNTLNSSPPLAPGEFDPNIDVYGIKCHENSPRKEVY
FMAIIDILTHYDAKKKAA HAAKTVKHGAGAEISTVNPEQYSKRFLDFIGHIL

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 60 mL TB supplemented with 8 g/l glycerol, 100 µg/mL kanamycin and 34 µg/mL chloramphenicol at 30 °C overnight. The overnight culture (60 mL) was used to inoculate 3 bottles containing each 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/mL kanamycin and approximately 200 µl 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,430 x g, 10 min, 4 °C). The resulting cell pellet (110 g wet cell weight) was resuspended in lysis buffer (1.5 mL/g cell pellet), supplemented with 6000 U Benzonase (Merck) and three tablets of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure
Columns IMAC: Ni-charged 5 mL HiTrap Chelating HP (GE Healthcare) Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare) Procedure Purification of the protein was performed as a two step process on an ÄKTAxpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. The filtered lysate was loaded onto the Ni-charged HiTrap Chelating column and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were pooled and fresh TCEP was added to a final concentration of 2 mM. The protein was subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 8.81 mg/mL in a volume of 1.5 mL. The detected mass (48310.9 Da) was very close to the expected mass (48313 Da) and the protein identity therefore confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the hanging drop vapour diffusion method in a 24-well plate. Prior setting-up the plate, protein solution was mixed with 2 mM Benzyl(1,3,5)triphosphate. Crystallization plate was immediately set-up afterwards. 0.6 µl protein solution (containing 2 mM Benzyl(1,3,5)triphosphate) (at 8.8 mg/mL) was mixed with 1.2 µl of well solution consisting of 16 % PEG3350 and 0.2 M Mg formate. The drop was incubated against a reservoir composed with 16 % PEG3350 and 0.2 M Mg formate. The plate was incubated at 4 °C and crystals appeared overnight. The crystals were quickly transferred to a cryo solution consisting of 19% PEG 3350, 0.25 M Mg formate, 25% Glycerol, 2mM Benzyl(1,3,5)triphosphate, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Final structure refinement done with 2.56 Å resolution data collected at DIAMOND, beamline I03 using a single wavelength (0.9792 Å)
Data Processing: The structure was solved by molecular replacement in PHASER using PDB entry 1BO1 as a molecular replacement probe. The space group was P21 with cell dimensions a=44.04 Å, b=98.51 Å, c=104.88 Å, β=93.43°. One dimer was located in the asymmetric unit. Coot was used for model building and at the final stages BUSTER was used for maximum-likelihood refinement. The final R values were: R=18.8% and Rfree=23.8%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2YBX.