INPP5B

PDB:3MTC

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:BC058932
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:
Tag:C-terminal hexahistidine tag
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:

MYTYIQNFRFFAGTYNVNGQSPKECLRLWLSNGIQAPDVYCVGFQELDLSKEAFFFHDTPKEEEWFKAVSEGLHPDAKYAKVKLIRL
VGIMLLLYVKQEHAAYISEVEAETVGTGIMGRMGNKGGVAIRFQFHNTSICVVNSHLAAHIEEYERRNQDYKDICSRMQFCQPDPSL
PPLTISNHDVILWLGDLNYRIEELDVEKVKKLIEEKDFQMLYAYDQLKIQVAAKTVFEGFTEGELTFQPTYKYDTGSDDWDTSEKCR
APAWCDRILWKGKNITQLSYQSHMALKTSDHKPVSSVFDIGVRVVAHHHHHH

Vector:pNIC-CH2

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 15 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 37 °C overnight. The overnight culture (15 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl Antifoam A204 (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 (30 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 2000 U Benzonase (Merck) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure
Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)
IEX column: MonoQ 5/50 GL (GE Healthcare)

Procedure
IMAC columns were equilibrated with IMAC wash1 buffer, and gel filtration columns were equilibrated with GF buffer. Purification of the protein was performed on an ÄKTAxpress system (GE Healthcare). The filtered lysate was loaded onto two Ni-charged HiTrap Chelating columns coupled in series 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 identified by SDS-PAGE, pooled, and fresh TCEP was added to a final concentration of 2 mM. The protein was concentrated using an Amicon Ultra-15 centrifugal filter device (10,000 NMWL; Millipore) to 23.7 mg/ml in a volume of 0.25 ml. The protein was diluted to 3 ml in MonoQ buffer A and further purified by IEX on a monoQ column by using a gradient of MonoQ buffer B from 0-30% in 20 column volumes. The protein eluted in 110 mM NaCl. The protein was concentrated as described above to 13.4 mg/ml in a volume of 0.30 ml. The identity of the protein was confirmed by mass spectrometry. To remove any bound metal ions from the proteins, dialysis against gel filtration buffer containing 25 mM ETDA was performed in a Slide-A-Lyzer 10 000 MW cutoff dialysis container (Pierce). Samples were subsequently dialyzed against gel filtration buffer without ETDA. The dialysed proteins were concentrated as described above to 21.7 mg/ml in a volume 0.040 ml.

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 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl protein solution (20.9 mg/ml) including 1 mM PtdIns(3,4,5)P3-dioctanoyl and 2 mM MgCl2 was mixed with 0.1 µl of well solution consisting of 0.02 M MgCl2, 0.1 M Hepes pH 7.5 and 22% polyacrylic acid 5100. The plate was incubated at 4 ºC and crystals appeared within 6 days. The crystals were quickly transferred to a cryo solution consisting of 0.02 M MgCl2, 0.1 M Hepes pH 7.5, 24% polyacrylic acid 5100, 1 mM PtdIns(3,4,5)P3-dioctanoyl, 2 mM MgSO4, 25% glycerol and 0.3 M NaCl, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 2.6 Å resolution was collected at ESRF beamline ID29.
Data Processing: The structure was solved by molecular replacement using the structure of apo-INPP5B as a search model (PDB entry 3N9V). The space group was $P2_13$ with cell dimensions a=b=c=133.76 Å. Refinement was performed using iterative cycles of manual model-building in Coot and maximum-likelihood refinement in Refmac. At the end of the refinement, R values reached 17.2% and 20.5% for R and R_{free}, respectively. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3MTC.