

INPP5B

PDB:3N9V

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:BC058932

Entry Clone Source:Mammalian Gene Collection

SGC Clone Accession:INPP5BA-k064

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:

MIQNFRFFAGTYNVNGQSPKECLRLWLSNGIQAPDVYCVGFQELDSKEAFFFHDTPKKEEWFKAVSEGLHPDAKYAKVKLIRLVGI
MLLLYVKQEHAAAYISEVEAETVGTGIMGRMGNGGVAIRFQFHNTSICVVNSHLAAHIEEYERRNQDYKDICSRMQFCQPDPSLPPL
TISNHDVILWLGDNLNRYIEELDVEKVKKLIIEKDFQMLYAYDQLKIQVAAKTVFEGFTEGELTFQPTYKYDTGSDDWDTSEKCRAPA
WCDRILWKGKNITQLSYQSHMALKTSDHKPVSSVFDIGVRVVNDEAHHHHHH

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 (~5 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 (28 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 ÄKTAexpress 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 19 mg/ml in a volume of 0.4 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 31.6 mg/ml in a volume of 0.22 ml. The identity of the protein was 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 sitting drop vapour diffusion method in a 96-well plate. 0.1 µl protein solution (31.6 mg/ml) including 2 mM biphenyl 2,3',4,5',6-pentakisphosphate was mixed with 0.1 µl of well solution consisting of 0.2 M MgCl₂, 0.1 M Tris pH 8.5, and 20% PEG 8000. The plate was incubated at 20 °C and crystals appeared within 10 days. The crystals were quickly transferred to a cryo solution consisting of 0.2 M MgCl₂, 0.1 M Tris pH 8.5, 22% PEG 8000, 25% glycerol and 0.3 M NaCl, and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Diffraction data to 2.8 Å resolution was collected at DIAMOND Light Source beamline I03.

Data Processing: The structure was solved by molecular replacement using a model based upon the structure of *Schizosaccharomyces pombe* synaptojanin as a probe (PDB entry 1I9Y). The space group was I2₁2₁2₁ with cell dimensions a=96.16 Å b=110.66 Å c=159.38 Å. Refinement was carried out using iterative cycles of manual building in Coot and maximum-likelihood refinement in Phenix. TLS refinement was used in the last steps using 2 TLS groups per monomer. At the end of the refinement, R values reached 19.1% and 23.1% for R and R_{free}, respectively. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3N9V.