INPP5E

PDB:2XSW

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:BC028032
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:INPP5EA-k040
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:

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MEGSLLASGALLGADELARYFPDRNVALFVATWNMQGQKELPPSLDEFLLPAEADYAQDLYVIGVQEGCSDRREWETRLQETLGPHY
VLLSSAAHGVLYMSLFIRRDLIWFCSEVECSTVTTRIVSQIKTKGALGISFTFFGTSFLFITSHFTSGDGKVAERLLDYTRTVQALV
LPRNVPDTNPYRSSAADVTTRFDEVFWFGDFNFRLSGGRTVVDALLCQGLVVDVPALLQHDQLIREMRKGSIFKGFQEPDIHFLPSY
KFDIGKDTYDSTSKQRTPSYTDRVLYRSRHKGDICPVSYSSCPGIKTSDHRPVYGLFRVKVRPGRDNIPLAAGKFDRELYLLGIKRR
ISAHHHHHH
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Vector:pNIC-CH2

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 28 °C overnight. The overnight culture (60 ml) was used to inoculate 3 x 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl Antifoam 204 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~3. 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 (115 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 3750 U HS-Nuclease and 3 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
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 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 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 10.2 mg/ml in a volume of 1.1 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 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 (10 mg/ml) including 2 mM MgCl2 and 1 mM PtdIns(3,4,5)P3-dioctanoyl was mixed with 0.1 µl of well solution consisting of 0.2 M MgCl2, 0.1 M Tris pH 7.0, 10% PEG 8000. The plate was incubated at 4 ºC and crystals appeared within 5 days. The crystals were quickly transferred to a cryo solution consisting of well solution complemented with 25% glycerol and 1 mM PtdIns(3,4,5)P3-dioctanoyl, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 1.8 Å resolution was collected at ESRF, beamline ID29.
Data Processing: The structure was solved by molecular replacement using *Schizosaccharomyces pombe* synaptojanin as a template (PDB entry 1I9Y). The space group was $P2_12_12_1$ with cell dimensions a=50.53 Å, b=103.58 Å and c=137.54 Å. Refinement was performed using iteratice cycles of manual building in Coot and maximum-likelihood refinement in PHENIX. TLS refinement using 3 TLS groups per monomer was employed in the final stages of refinement. At the end of the refinement, R values reached 16.65% and 19.47% for R and R_{free}, respectively. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2XSW.