BPNT1

PDB:2WEF

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|116812595
Entry Clone Source:NEDO
SGC Clone Accession:BPNT1A-k016
Tag:C-terminal hexahistidine tag *ahhhhhh
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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MTVLMRLVASAYSIAQKAGMIVRRVIAEGDLGIVEKTCATDLQTKADRLAQMSICSSLARKFPKLTIIGEEDLPSEEVDQELIEDSQ
WEEILKQPCPSQYSAIKEEDLVVWVDPLDGTKEYTEGLLDNVTVLIGIAYEGKAIAGVINQPYYNYEAGPDAVLGRTIWGVLGLGAF
GFQLKEVPAGKHIITTTRSHSNKLVTDCVAAMNPDAVLRVGGAGNKIIQLIEGKASAYVFASPGCKKWDTCAPEVILHAVGGKLTDI
HGNVLQYHKDVKHMNSAGVLATLRNYDYYASRVPESIKNALVP*ahhhhhh
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Vector:pNIC-CH2

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 20 mL TB supplemented with 8 g/l glycerol, 100 µg/mL kanamycin and 34 µg/mL chloramphenicol at 30 °C overnight. 20 mL of the overnight culture were used to inoculate 1.5 l TB supplemented with 50 µg/mL kanamycin and approximately 500 µl PPG P2,000 81380 anti-foam solution (Fluka). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C for 4 hours after which the bottle 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 (4,400 x g, 10 min, 4 °C). The resulting cell pellet (19 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)
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 two HiTrap Chelating columns connected in series and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC columns 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 with 10,000 NMWL (Millipore) to 47.6 mg/mL in a volume of 0.22 mL.

Extraction

Procedure
The cell suspension was quickly thawed in water and diluted to 2 x 68 mL with lysis buffer. 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
Adenosine monophosphate (AMP), Magnesium ions, Phosphate**MassSpec:**
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method using a 96-well plate. 0.1 µl of the protein sample (diluted to 15 mg/mL) including 5 mM LiSO4, 5 mM MgCl2 and 2mM 3′-phosphoadenosine 5′-phosphate (PAP) was mixed with 0.1 µl of well solution consisting of 0.1 M Bis-Tris pH 5.5, 0.4 M magesium chloride hexahydrate, 32.5% PEG 3350. The plate was incubated at 4 ºC and crystals appeared within 5 days. Cryo solution containing 0.2 M Magnesium chloride, 0.1 M Bis-Tris pH 5.5, 32.5% PEG 3350, 0.3 M NaCl and 20% glycerol was added directly to the drop. Crystals were mounted and flash-frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data to 1.8 Å resolution was collected from a single crystal at DIAMOND (I02).
Data Processing: Crystal belonged to P2 21 21 space group with cell parameters of a=38.98 Å, b=67.66 Å, c=100.4 Å. The asymmetric unit contains one monomer. The structure was solved by molecular replacement using MOLREP with rat BPNT1 protein structure (2JP4) as a search model. Adenosine monophosphate (AMP) and phosphate (PO4) likely originating from 3′-phosphoadenosine 5′-phosphate (PAP) was present in the active site together with Magnesium ions. Structure was refined with REFMAC5. Final R-values were R=14.4% and Rfree=18.9%. Coordinates and structure factors are deposited to PDB with accession code 2WEF.