GMPS

PDB:2VPI

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:BC012178
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
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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mhhhhhhssgvdlgtenlyfq*smEGAVVILDAGAQYGKVIDRRVRELFVQSEIFPLETPAFAIKEQGFRAIIISGGPNSVYAEDAP
WFDPAIFTIGKPVLGICYGMQMMNKVFGGTVHKKSVREDGVFNISVDNTCSLFRGLQKEEVVLLTHGDSVDKVADGFKVVARSGNIV
AGIANESKKLYGAQFHPEVGLTENGKVILKNFLYDIAGCSGTFTV
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Vector:pNIC-Bsa4

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 ºC overnight. The overnight culture (20 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 0.2 ml PPG P2,000 81380 anti-foam solution (Fluka). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~2. The bottle was down-tempered to 18 ºC 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 ºC). The resulting cell pellet (26.7 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 ºC.

Purification

Procedure
Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 75 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 concentrated using an Amicon centrifugal filter device with 10,000 NMWL (Millipore). When the protein had reached a concentration of 7.9 mg/ml in a volume of 0.3 ml the concentration was stopped due to signs of precipitation and frozen at -80 ºC .

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 (49,100 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 of the protein solution (7.9 mg/ml), 10 mM ATP and 10 mM MgCl2 was mixed with 0.1 µl of well solution consisting of 0.1 M Bis-Tris pH 5.5, 0.2 M ammonium acetate and 25% PEG 3350. The plate was incubated at 20 ºC and rod shaped crystals appeared between 2 and 7 days. The crystal was quickly transferred to cryo solution consisting of 0.1 M Bis-Tris, pH 5.5, 0.2 M ammonium acetate, 28% PEG 3350, 0.3M NaCl and 20% glycerol, and flash-frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data was collected at ESRF (ID23-2) at 100K. 200 frames were collected with 2º oscillation range.
Data Processing: The crystal belonged to space group P21 with the cell parameters a = 35.7 Å, b = 120.9 Å, c= 47.3 Å, α = γ = 90º β = 106.4º. Data was processed and scaled using XDS and XSCALE. The structure was solved by molecular replacement with MOLREP using the structure of the glutaminase of the *E.coli* structure (pdb-code:1GPM) as search model. Model building and refinement were performed in COOT and REFMAC5. Data in the interval 20 Â□ 2.4 Å resolution was used and at the end of the refinement the R values were R=20.6% and Rfree=26.1%. The coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 2VPI.