UMPS

PDB:2WNS

Revision

Revision Type:created
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Entry Clone Accession:BC000364
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:UMPSA-k015
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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MALGPLVTGLYDVQAFKFGDFVLKSGLSSPIYIDLRGIVSRPRLLSQVADILFQTAQNAGISFDTVCGVPYTALPLATVICSTNQIP
MLIRRKETKDYGTKRLVEGTINPGETCLIIEDVVTSGSSVLETVEVLQKEGLKVTDAIVLLDREQGGKDKLQAHGIRLHSVCTLSKM
LEILEQQKKVDAETVGRVKRFIQEAHHHHHH
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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 °C 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 200 µl 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C. 3 hours after inoculation the culture was down-tempered to 18 °C over a period of 1 hour and then 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 (21 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 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 subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 29.7 mg/ml in a volume of 0.7 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
OROTIDINE 5'-MONOPHOSPHATE (OMP)**MassSpec:**
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. Protein solution was diluted to 14.8 mg/ml and supplemented with 5 mM OMP and 5 mM MgCl2, final concentrations. Then, 0.1 µl protein solution was mixed with 0.2 µl of well solution consisting of 0.1 M MMT pH 7 and 25% PEG 1500. The plate was incubated at 22 ºC and crystals appeared in two days. The crystals were quickly transferred to a cryo solution consisting of 0.1 M MMT pH 7, 26% PEG 1500, 20% glycerol and 0.3 M NaCl, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 1.9 Å resolution was collected at ESRF ID23-1.
Data Processing: The structure was solved by molecular replacement using a truncated version of 2YZK as template. The space group was P21 with the cell dimensions a=64.4 Å, b=43.6 Å, c=71 Å, a=g=90°, b=113.4°. Initial model was build using ARP-WARP. REFMAC5 was used for refinement and Coot for final model building. The structure contained 2 molecules in the asymmetric unit and OMP and Mg2+ was found ordered. Coordinates for the crystal structure were deposited in the Protein Data Bank under the accession code 2wns.