PARP14

PDB:3GOY

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:
Entry Clone Source: Synthetic gene
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:

mhhhhhhssgvdlgtenlyfq*smDMKQQNFCVVELLPSDPEYNTVASKFNQTCSHFRIEKIERIQNPDLWNSYQAKKKTMDAKNGQ
TMNEKQLFHGTDAGSVPHVNRNGFNRSYAGKNAVAYGKGTYFAVNANYSANDTYSRPDANGRKHVYYVRVLTGIYTHGNHSLIVPPS
KNPQNPTDLYDTVTDNVHHPSLFVAFYDYQAYPEYLITFRK

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 (15 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 750 µl 204 Antifoam A6426 (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 (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (32 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 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, 10,000 NMWL (Millipore) to 14.2 mg/ml in a volume of 1.0 ml. The identity of the protein was confirmed by mass spectrometry.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease at a molar ratio of 30:1 at 4 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The buffer was changed to GF buffer containing 2 mM TCEP and the protein was concentrated using a Vivaspin 20 centrifugal filter device with 10,000 MWCO (Sartorius). The final protein concentration was determined to 13.8 mg/ml in a volume of 0.20 ml.

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 (49,100 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. 5 mM 3-aminobenzamide was added to 200 µl of the protein solution (13.8 mg/ml), and the sample was concentrated to 50 µl. 0.1 µl of this protein solution was mixed with 0.1 µl of well solution consisting of 0.2 M sodium malonate and 20% (w/v) PEG 3350. The plate was incubated at 20 ºC and crystals appeared within one month. The crystals were quickly transferred to a cryo solution consisting of 0.2 M Sodium Malonate, 22% PEG 3350, 0.2 M NaCl and 20% PEG 400, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 2.8 Å resolution was collected at DIAMOND beamline I03.
Data Processing: The structure was solved by molecular replacement using the PARP-15 structure as template (PDB: 3BLJ). The space group was C2 with cell dimensions a=82.75 Å b=144.27 Å c=79.70 Å, β=100.55°. Four monomers were located in the asymmetric unit. Analysis of the data revealed pseudotranslation, and was considered in the molecular replacement step using Molrep software. PHENIX was used for refinement and Coot for model building. Data

in the interval 25.0-2.80 Å resolution was used and at the completion of refinement R = 25.6% and Rfree = 29.2%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3GOY.