Molecular Biology

Entry Clone Accession: IMAGE:4719195
Entry Clone Source: MGC
SGC Construct ID: RG9MTD1A-c113
Protein Region: Q203-T403
Vector: pNIC-CTHF
Tag: C-TEV;C-6HIS
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MQPLVFDMAYENYMKRKELQNTVSQLLESEGWNRRNVDPFHIYFCNLKIDGALHRELV
KRYQEKWDKLLLTSTEKSHVDLFPKDSIIYLTADSPNVMTTFRHDKVYVIGSFVDKSMQP
GTSLAKAKRLNLATECLPLDKYLQWEIGNKNLTLDQMIRILLCLKNNGNWQEALQFVPK
RKHTGFLEISQHSQEFINRLKKAKTAENLYFQSHHHHHHDYKDDDDK
Sequence after tag cleavage:
MQPLVFDMAYENYMKRKELQNTVSQLLESEGWNRRNVDPFHIYFCNLKIDGALHRELV
KRYQEKWDKLLLTSTEKSHVDLFPKDSIIYLTADSPNVMTTFRHDKVYVIGSFVDKSMQP
GTSLAKAKRLNLATECLPLDKYLQWEIGNKNLTLDQMIRILLCLKNNGNWQEALQFVPK
RKHTGFLEISQHSQEFINRLKKAKTAENLYFQ
DNA Sequence:
CTTAAGAAGGAGATATACTATGCAACCTTTGGTTTTTGACATGGCTTACGAAAATTATA
TGAAACGAAAAGAATTGCAGAATACTGTTTCCCAGCTTTTAGAAAGTGAAGGATGGA
ACAGAAGAAATGTTGATCCTTTCCATATTTATTTCTGCAATCTAAAAATAGATGGTGCTT
TGCACAGAGAGTTAGTTAAACGGTATCAAGAAAAATGGGACAAATTGCTTTTAACATC
AACAGAAAAGTCTCATGTAGATTTATTTCCAAAGGACAGTATTATCTATTTAACTGCAG
ATTCTCCCAATGTTATGACTACTTTCAGGCATGACAAAGTTTATGTAATTGGGTCTTTTG
TTGATAAGAGTATGCAGCCAGGCACATCCCTAGCCAAGGCAAAACGGCTGAACCTGG
CAACTGAATGCCTTCCATTAGATAAATATTTACAATGGGAAATTGGTAACAAAAATCTC
ACCTTAGATCAAATGATACGTATTTTGTTATGTCTGAAAAACAATGGTAATTGGCAAGA
GGCTCTGCAATTCGTTCCCAAGAGAAAACATACTGGTTTTCTGGAGATTTCTCAGCAT
TCTCAAGAGTTTATCAACAGACTAAAGAAGGCAAAGACTGCAGAGAACCTCTACTTC
CAATCGCACCATCATCACCACCATGATTACAAGGATGACGACGATAAGTGAGGATCC

Protein Expression

Medium: Terrific Broth
Antibiotics: Kanamycin
Procedure: Plasmids were transformed into *E. coli* BL21(DE3)-R3-pRARE2 competent cells, cultured in Terrific Broth at 37°C, and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for overnight growth at 18^{o}C.

Protein Purification

Buffers:
Lysis buffer: 500 mM NaCl, 50 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP, 20 mM imidazole
Wash buffer: 500 mM NaCl, 50 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP, 40 mM imidazole
Elution buffer: 500 mM NaCl, 50 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP, 250 mM imidazole

SEC buffer: 500 mM NaCl, 50 mM HEPES pH 7.5, 5% glycerol, 0.5 mM TCEP

Procedure:
Cell lysis
Frozen cell pellets were thawed and resuspended on ice in 50 mL lysis buffer containing 1 mL protease inhibitor cocktail set III (Calbiochem) per L of harvested cell culture. The cells were lysed by passing five times on a homogeniser (EmulsiFlex-C5-Avestin) to a pressure of 1000 bar. The cell lysate was clarified by centrifugation (Beckman Coulter Avanti JXN-26) at 4°C for 1 h at 16500 rpm.

Immobilised metal affinity chromatography (IMAC)
Clarified lysate from large-scale extraction was added with 2 mL of Ni-NTA affinity resin (GE Healthcare) and incubated at 4°C for 1 h rotating at 8 rpm. The lysate, together with Ni-NTA resin, was centrifuged (Beckman Coulter Avanti JXN-26) for 30 min at 5000 rpm at 4°C. The Ni-NTA pellet was applied onto a 1.5x10 cm (DxH) column (Bio-Rad) under gravity flow and the flow-through was collected for SDS-PAGE analysis. The Ni-NTA resin was washed with 2 x 10 CVs Lysis buffer and 2 x 10 CVs Wash buffer. The bound protein was eluted with 5 x 2 CVs of Elution buffer.

Size exclusion chromatography (SEC)
Pooled fractions from IMAC were concentrated to 5 mL using an Amicon Ultra centrifugal filter device (Merck Millipore) with a 10 kDa cut-off. The concentrated sample was loaded onto a 120 mL Sephadex 75 HiLoad 16/60 column connected to an AKTA-Express (GE Healthcare) in 4°C. Before loading the protein, the column was pre-equilibrated with SEC buffer. Protein was loaded and eluted at a rate of 1 mL/min and 1.8 mL fractions were collected throughout the run.

Treatment with Tobacco etch virus protease
To remove the affinity tag from target proteins, Tobacco etch virus (TEV) protease was added to the eluates in a 1:20 (w/v) protease-to-protein ratio and the mixture was incubated overnight at 4°C.

Reverse IMAC
TEV protease-treated sample was applied to a 2x10 cm gravity column containing 125 µL Ni-NTA affinity resin (GE Healthcare) equilibrated with Lysis buffer. The Ni-NTA resin was washed with 2 x 8 CVs Binding buffer and 2 x 8 CVs Wash buffer. Fractions containing protein were pooled.

Concentration: 10.93 mg/ml
Mass-spec Verification: Expected mass of the construct confirmed

Structure Determination

Crystallization: Crystals of hMRPP1$_{MT}$ were grown by vapour diffusion at 4°C, from sitting drops mixing 200 nL of protein (11 mg/ml; pre-incubated with 4.8 µM SAM) and 100 nL of reservoir solution containing either 14% PEG 1000, 28% glycerol or 12% PEG 1000, 28% glycerol, 1.5%(w/v) PEG 3350. Crystals were cryo-protected with reservoir solution supplemented with 25% (v/v) propylene glycol, and flash-cooled in liquid nitrogen. Heavy atom-derivatised crystals were obtained by soaking with 10mM $C_9H_9HgNaO_2S$ for 1h10min.

Data Collection: Diffraction data were collected on the Diamond Light Source beamline I04-1 for the HA-soaked crystals to 2.49 Å, and for two crystals of the as-purified protein to 2.22 Å and 1.96 Å.

Data Processing: Phases were determined by single-wavelength anomalous dispersion (SAD) with the SHELX package using the HA dataset. The phases from the HA-dataset were combined with the 2.22 Å native dataset, and the model was built in COOT. This model was used as search model in a molecular replacement done in PHASER from the CCP4 Suite using the native data collected to 1.96 Å. Modelling and refinement were carried out using Refmac and COOT.