Molecular Biology

Entry Clone Accession: MGC:22457 IMAGE:4767736
Entry Clone Source: MGC
SGC Construct ID: PAHA-c011
Protein Region: G19-V118
Vector: pNIC28-Bsa4
Tag: N-6HIS; TEV cleavage site
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MHHHHHHSSGVDLGTENLYFQSMGQETSYIEDNCNQNGAISLIFSLKEEVGALAKVLRL
FEENDVNLTHIESRPSRLKKDEYEFFTHLDKRSLPALTNIIKILRHDIGATVHELSRDKKKD
TV
Sequence after tag cleavage:
SMGQETSYIEDNCNQNGAISLIFSLKEEVGALAKVLRLFEENDVNLTHIESRPSRLKKDEY
EFFTHLDKRSLPALTNIIKILRHDIGATVHELSRDKKKDTV
DNA sequence:
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTA
CTTCCAATCCATGGGACAGGAAACAAGCTATATTGAAGACAACTGCAATCAAAATGGT
GCCATATCGCTGATCTTCTCACTCAAAGAAGAAGTTGGTGCATTGGCCAAAGTATTGC
GCTTATTTGAGGAGAATGATGTAAACCTGACCCACATTGAATCTAGACCTTCTCGTTTA
AAGAAAGATGAGTATGAATTTTTCACCCATTTGGATAAACGTAGCCTGCCTGCTCTGA
CAAACATCATCAAGATCTTGAGGCATGACATTGGTGCCACTGTCCATGAGCTTTCACG
AGATAAGAAGAAAGACACAGTGTGACAGTAAAGGTGGATACGGATCCGAA

Protein Expression

Medium: Terrific Broth
Antibiotics: Kanamycin
Procedure: plasmid was transformed into *E. coli* BL21(DE3), cultured in Terrific Broth at 37^{o}C until OD_{600} ~1.5, and induced with 0.5 mM IPTG for overnight growth at 18°C.

Protein Purification

Procedure: Cells were harvested and homogenized in buffer A (50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 0.5 mM TCEP, EDTA-free protease inhibitor). Insoluble debris was removed by further centrifugation. Proteins were purified by passing cell extracts through a 1ml HisTrap column pre-equilibrated with buffer A, and eluted with buffer B (buffer A + 250 mM Imidazole). Eluted fractions were treated with TEV protease at a protein:protease ratio of 1:20, and incubated overnight at 4°C in order to cleave off the His_6-tag. The tag-cleaved protein was applied onto a 1ml HisTrap column, pre-equilibrated with GF buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 5% glycerol and 0.5 mM TCEP). The flow-through sample was applied onto a HiLoad 16/60 Superdex 75 column pre-equilibrated with GF buffer.

Concentration: 13.5 mg/ml
Mass-spec Verification: Yes

Structure Determination

Crystallization: Protein was concentrated to a 13.5mg/ml, where l-Phenylalanine (Phe) was added to a final concentration of 10mM. The sample was treated with trypsin at a protein:trypsin mass ratio of 1:100 immediately prior to crystallization set up. Crystals were grown by vapour diffusion in sitting drops at 20°C. A sitting drop consisting of 100 nl trypsin-treated protein and 200 nl well solution was equilibrated against well solution containing 25% PEG 3350, 0.20 M NaCl and 0.1 M BIS-Tris pH 5.5. The crystals were mounted directly from the drop using 25% ethylene glycol as a cryoprotectant and flash-cooled in liquid nitrogen.

Data Collection: Diffraction data was collected at the Diamond Light Source *beamline* Dmnd I03; *Resolution:* 1.8 Å
Data Processing: Diffraction data were processed with the CCP4 program suite. The structure was solved by molecular replacement using the program PHASER with the *Chlorobium tepidum* prephenate dehydratase structure (PDB code 2QMX) as search model. Iterative cycles of restrained refinement and manual model building were performed using COOT and PHENIX.