ADSL

PDB:2VD6

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:BC000253 (but contains a point mutation introduced by PCR shown in bold)
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) (Novagen)

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smAAGGDHGSPDSYRSPLASRYASPEMCFVFSDRYKFRTWRQLWLWLAEAEQTLGLPITDEQI**Q**E
MKSNLENIDFKMAAEEEKRLRHDVMAHVHTFGHCCPKAAGIIHLGATSCYVGDNTDLIILRNALDLLLPKLARVISRLADFAKERAS
LPTLGFTHFQPAQLTTVGKRCCLWIQDLCMDLQNLKRVRDDLRFRGVKGTTGTQASFLQLFEGDDHKVEQLDKMVTEKAGFKRAFII
TGQTYTRKVDIEVLSVLASLGASVHKICTDIRLLANLKEMEEPFEKQQIGSSAMPYKRNPMRSERCCSLARHLMTLVMDPLQTASVQ
WFERTLDDSANRRICLAEAFLTADTILNTLQNISEGLVVYPKVIERRIRQELPFMATENIIMAMVKAGGSRQDCHEKIRVLSQQAAS
VVKQEGGDNDLIERIQVDAYFSPIHSQLDHLLDPSSFTGRASQQVQRFLEEEVYPLLKPYESVMKVKAE

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 20 ml TB supplemented with 8 g/l glycerol and 100 µg/ml kanamycin 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 200 µl BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~2.5. The culture 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 (5,500 x *g*, 10 min, 4 ºC). The resulting cell pellet (27.4 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet), supplemented with 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 200 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. Some precipitation was observed in the sample and removed by centrifugation. The protein was subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 10,000 NMWL (Millipore) to 12 mg/ml in a volume of 4.0 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water and 2000 U Benzonase (Merck) was added. 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
MassSpec:
Crystallization: Crystals were obtained by the hanging drop vapour diffusion method in a 24-well plate containing 500 µl well solution. 1 µl of the protein solution (diluted to 5.5 mg/ml) including 10 mM AMP was mixed with 1 µl of well solution consisting of 0.2 M potassium formate and 14% PEG 3350. The plate was incubated at 4 ºC. The crystals were soaked for 45 minutes in cryo solution consisting of 10 mM AMP, 50 mM fumarate, 0.1 M potassium formate, 17% PEG 3350, 0.2 M NaCl, and 25% glycerol, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data to 2.3 Å resolution was collected from a single crystal at ESRF (ID23-1). The crystal belonged to space group P21 21 21 with cell parameters of a=87.3 Å, b=128.1 Å, c=190.5 Å. The asymmetric unit contained a tetramer.
Data Processing: The Xray data was processed by XDS and scaled by XSCALE. The structure was solved by molecular replacement using MOLREP with the apo structure as search model (2J91). The crystals were soaked with the AMP and fumarate products, however in two subunits of the tetramer the covalent bond of the N6-(1,2-dicarboxyethyl)AMP substrate was formed. The MR solution was further refined with REFMAC5. Final R-values were R=19.2% and Rfree=22.9%. Coordinates and structure factors were deposited to the protein data bank with accession code 2VD6.