LASP1

PDB:3I35

Revision

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Entry Clone Accession:gi|5453710
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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:

MHHHHHHSSGVDLGTENLYFQSMAPGGGGKRYRAVYDYSAADEDEVSFQDGDTIVNVQQIDDGWMYGTVERTGDTGMLPANYVEAI

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 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 (16 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 Ni-charged HiTrap Chelating columns connected in series 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 total volume of the pooled fractions was 12 ml and the concentration of the protein 1.52 mg/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 in a molar ratio of 50:1 at 8 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 HisTrap HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a Vivaspin 20 centrifugal filter device with 10,000 MWCO (Sartorius). The final protein concentration was determined to 9.35 mg/ml in a volume of 0.68 ml The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water bath. 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).
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl protein solution (9.35 mg/ml) was mixed with 0.2 µl of well solution consisting of 1.6 M tri-sodium citrate dihydrate pH 6,5. The plate was incubated at 20 ºC. The crystals were quickly transferred to a cryo solution consisting of well solution complemented with 20% D-glucose and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection:
Data Processing: Data was integrated using XDS and scaled using XSCALE. The structure was solved by molecular replacement using MOLREP with the cortactin-SH3 domain (PDB: 2D1X). The space group was P 1 2 1 with cell dimensions a= 25.35 Å b= 23.24 Å c= 44.72Å, β=92.47°. One monomer was located in the asymmetric unit. REFMAC5 was used for final refinement and Coot for model building. Data in the interval 23.24-1.40 Å resolution was used and at the end of the refinement the R values were: R=20.2% and Rfree=22.2%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3I35 .