

LMSAH

PDB:3G1U

Revision

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Entry Clone Accession:Q4Q124

Entry Clone Source:in-house cloning

SGC Clone Accession:LMSAHA-k006

Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site:

mhhhhhssgvdlgtenlyfq*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:

mhhhhhssgvdlgtenlyfq*smADYKVKDISLAEWGRKAIELAENEMPGLMELRREYGPSQPLKGAKIAGCLHMTVQTAVLIETL
KALGAELRWSSCNIFSTQDNAAAAIAKTGVPVFAWKGETDEEYEWCIQTVKGFSGDGLPNMILDDGGDLTNLVIDRYPELVPKIFG
ISEETTTGVKNLYKRLSKGNLPISAINVND SVTKSKFDNLYGCRESLVDGIKRATDVMIAGKTCCVCGYGDVGKGCAALRAFGARV
VVTEVDPINALQASMEGYQVALVEDVMADAHIFVTTTGND DIITSDHFPHMRDDAIVCNIGHFDTEIQVGWLEANAKEHVEIKPQVD
RYTMENGRHIILLAKGRLVNLGCASGHPSFVMSNSFTNQVLAQIELWSNRDNGKYPRGDKAGVFFLPKALDEKVAALHLAHVGAKLT
KLTPKQAEYINCPVNGPFFK

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 0.75 ml 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The bottle 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 (20 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 200 Prep Grade (GE Healthcare)

Procedure

Purification of the protein was performed as a two step process on an ÄKTAexpress 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. The protein was subsequently concentrated using an Amicon centrifugal filter device with 10,000 NMWL (Millipore) to 25 mg/ml in a volume of 2.4 ml.

Tag removal

The N-terminal histidine tag was proteolytically removed by incubating LMSAHA with His-tagged TEV protease (van den Berg, S., *J. Biotech* **121**, 291-298 (2006)) at a molar ratio of 160:1 at 20 °C 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 cleaved protein was concentrated using a centrifugal filter device to 35 mg/ml, resulting in a volume of 0.5 ml, and subsequently dialyzed against GF buffer containing 2 mM TCEP. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure

The cell suspension was briefly 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 °C). The supernatant was decanted and filtered through 0.45 µm flask filter.

Concentration:

Ligand

MassSpec:

Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl of the protein solution (diluted to 25 mg/ml with GF buffer) including 3 mM NAD was mixed with 0.1 µl of well solution consisting of 0.1 M Bicine, pH 9 and 20% PEG 6000. The plate was incubated at 4 °C and crystals appeared within 1 day. The crystals were quickly transferred to cryo solution containing well solution, 0.2 M NaCl and 20% glycerol and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Data sets were collected on a single crystal to 2.2 Å resolution at DIAMOND (I03). This data used for the final refinement belonged to P1 space group with cell parameters of $a = 72.38 \text{ Å}$, $b = 82.47 \text{ Å}$, $c = 83.88 \text{ Å}$ $\alpha = 87.02^\circ$ $\beta = 71.41^\circ$ $\gamma = 74.00^\circ$

Data Processing: Data was integrated with XDS, scaled with XSCALE and the structure was solved using MOLREP with PDB ID = 1LI4 as a search model. Four chains were found in the asymmetric unit. The model was improved by successive rounds of manual model building in

COOT and refinement with Refmac5. Final R-values were R= 18.15% and Rfree= 23.16%. Coordinates and structure factors were deposited in the PDB with accession code 3G1U.