KIAA0828

PDB:3GVP

Revision

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Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:BL21(DE3) gold pRARE2

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smKQQKNSKGSSDFCVKNIKQAEFGRREIEIAEQEMPALMALRKRAQGEKPLAGAKIVGCTHITA
QTAVLMETLGALGAQCRWAACNIYSTLNEVAAALAESGFPVFAWKGESEDDFWWCIDRCVNVEGWQPNMILDDGGDLTHWIYKKYPN
MFKKIKGIVEESVTGVHRLYQLSKAGKLCVPAMNVNDSVTKQKFDNLYCCRESILDGLKRTTDMMFGGKQVVVCGYGEVGKGCCAAL
KAMGSIVYVTEIDPICALQACMDGFRLVKLNEVIRQVDIVITCTGNKNVVTREHLDRMKNSCIVCNMGHSNTEIDVASLRTPELTWE
RVRSQVDHVIWPDGKRIVLLAEGRLLNLSCSTVPTFVLSITATTQALALIELYNAPEGRYKQDVYLLPKKMDEYVASLHLPTFDAHL
TELTDEQAKYLGLNKNGPFKPN

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 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 flasks were 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 pellets (20 g wet cell weight) were resuspended in lysis buffer (1.5 mL/g cell pellet), supplemented with 1000 U Benzonase (Merck) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science) per 30 mL lysis buffer. 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 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. The protein used in crystallisation trials was obtained from two different protein batches. The first (Batch1) was concentrated using an Amicon centrifugal filter device with 10,000 NMWL (Millipore) to 41.8 mg/ml in a volume of 1.8 mg/ml, while the second (Batch 2) to 10.0 mg/mL in a volume of 6.0 mL.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating either of the KIAA0828A batches with His-tagged TEV protease (van den Berg, S., J. Biotech 121, 291-298 (2006)) at a molar ratio of 160:1 at 20 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target proteins were 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 proteins were concentrated using a centrifugal filter device to 37.4mg/mL (1mL) and 27.6 mg/mL (2mL), for Batch1 and 2 respectively. 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 ºC). The supernatant was decanted and filtered through 0.45 µm flask filter.
Concentration:
Ligand
NAD**MassSpec:**
Crystallization: The first crystal (Crystal 1, obtained from Batch1) was obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl of the protein solution (diluted to 28.8 mg/mL with GF buffer) including 4 mM NAD was mixed with 0.1 µl of well solution consisting of 0.16 M sodium malonate and 22% PEG 3350. The plate was incubated at 4 ºC and crystals appeared within 5 days. The diffracting crystal was quickly transferred to cryo solution containing well solution, 0.2 M NaCl and 20% glycerol and flash frozen in liquid nitrogen. The second crystal (Crystal 2, obtained from Batch 2) was obtained by the hanging drop vapour diffusion method in a 24-well plate. 1 µl of the protein solution (diluted to 21.8 mg/mL with GF buffer) including 4 mM NAD was mixed with 2 µl of well solution consisting of 0.1 M HEPES pH 7.5, 0.2 M sodium malonate and 12.5% PEG 3350. The plate was incubated at 4 ºC and crystals appeared within 5 days. The diffracting crystal was transferred to a dehydration solution containing well solution plus 20% PEG 4K for 10 minutes. The crystal was then cryo-cooled in a solution containing well solution and 20% ethylene glycol by flash frozing in liquid nitrogen.
NMR Spectroscopy:

Data Collection: The data set collected on Crystal 1 was to a 3.2 Å resolution at the ESRF (ID23-1) . This data used for generating the initial model belonged to P 41 21 2 space group with cell parameters of a=124.76 Å, b=124.76 Å, c=567.25 Å α=90.00 β=90.00 γ=90.00. Data sets collected on the second crystal were to a 2.25 Å resolution at DIAMOND (I02). This data used for the final refinement also belonged to P 41 21 2 space group with cell parameters of a=164.19 Å, b=164.19 Å, c=184.61 Å α=90.00 β=90.00 γ=90.00

Data Processing: The first data set (obtained from Crystal 1) was integrated with XDS, scaled with XSCALE and the structure was solved using PHASER with PDB ID = 3D64 as a search model. Four chains were found in the asymetric unit. The model was rebuilt using NCS averaged maps in CNS. This model was improved by successive rounds of manual model building in COOT and refinement with Refmac5. This result was used as a search model for the second data set (obtained from Crystal 2). This data was integrated with XDS, scaled with SCALA and the structure was solved using PHASER. This final model was improved by successive rounds of manual model building in COOT and refinement with Refmac5. Final R-values were R= 22.6% and Rfree=18.2%. Coordinates and structure factors were deposited in the PDB with accession code 3GVP