BCL2A1

PDB:2VM6

Revision

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Revised by:created
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Entry Clone Accession:BC016281
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*smTDCEFGYIYRLAQDYLQCVLQIPQPGSGPSKTSRVLQNVAFSVQKEVEKNLKSCLDNVNVVSV
DTARTLFNQVMEKEFEDGIINWGRIVTIFAFEGILIKKLLRQQIAPDVDTYKEISYFVAEFIMNNTGEWIRQNGGWENGFVKKFE

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:Native protein
Cells from glycerol stock were grown in 50 ml LB, supplemented with 50 µg/ml kanamycin and grown at 37 ºC overnight. The overnight culture was used to inoculate 3 x 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl PPG P2,000 81380 anti-foam solution (Fluka). The cultures were grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~2. The cultures were down-tempered to 18 ºC over a period of 1 hour. Expression of target protein was induced by addition of 0.5 mM IPTG and allowed to continue overnight. Cells were harvested the following morning by centrifugation (3,800 x *g*, 10 min, 4 ºC). The resulting cell pellet (90 g wet cell weight) was resuspended in lysis buffer (2 ml/g cell pellet) supplemented with two tablets of Complete EDTA-free protease inhibitor (Roche Applied Science), 2000 U Benzonase (Merck) and a knife edge of lysozyme (Sigma), and stored at -80 ºC.
SeMet enriched protein
Cells from glycerol stock were grown in 300 ml LB supplemented with 100 µg/ml kanamycin at 37 ºC overnight. 240 ml of the overnight culture was used to inoculate 12 bottles with 1.5 l minimal medium (without amino acids) supplemented with 50 µg/ml kanamycin and approximately 200 µl PPG P2,000 81380 anti-foam solution (Fluka) per bottle. The cultures were

grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 ºC until OD600 reached ~0.6. The cultures were down-tempered to 18 ºC and amino acids were added (Van Duyne, G. D., *J. Mol. Biol.* **229**, 105-124 (1993)). After 20 minutes, expression of target protein was induced by addition of 0.5 mM IPTG and expression was allowed to continue at 18 ºC overnight. Cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 ºC). The resulting cell pellet (17 g from 18 liter culture) 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 75 Prep Grade (GE Healthcare)

Procedure
Purification of the native 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 subsequently concentrated in a centrifugal filter device. Purification of the SeMet enriched protein was performed in basically the same way. The filtered lysate was purified by IMAC and gel filtration chromatography followed by a concentration step. The identities of the proteins were confirmed by mass spectrometry and selenomethionine was fully incorporated at all 3 methionine positions in the labeled protein.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease. After purification the cleaved native protein was concentrated to 8.0 mg/ml in a volume of 0.2 ml. The histidine tag removal of SeMet-labeled protein was achieved by incubating His-tagged TEV protease and protein in a 1:20 molar ratio at 4 ºC overnight. The cleaved protein was purified from tag and protease by incubating the reaction mix with Ni-NTA agarose. The cleaved protein retrieved from the supernatant was concentrated in a Vivaspin 20 centrifugal filter device with 5,000 MWCO (Sartorius) to a final concentration of 20 mg/ml in 1.5 ml.

Extraction

Procedure
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (VibraCell, Sonics) at 80% amplitude for 3 min effective time (puls 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 40 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter. For SeMet-labeled protein, the cell pellet was quickly thawed and resuspended in lysis buffer supplemented with Complete EDTA-free protease inhibitor (Roche Applied Science) and Benzonase (Merck). The cell suspension was then treated in the same as descibed for the unlabeled protein.
Concentration:
Ligand
MassSpec:

Crystallization: Native crystals were obtained by the hanging drop vapour diffusion method in a 24-well plate containing 500 µl well solution. Prior to crystallization the Bim-BH3 peptide was dissolved in gel filtration buffer to 25 mM and added to the protein solution in a 1:1 molar ratio. 1 µl of the protein sample (8 mg/ml) including peptide was mixed with 1 µl of well solution consisting of 0.1 M bis-Tris, pH 5.8 and 2.0 M ammonium sulfate. The plate was incubated at 20 ºC. The crystals were quickly transferred to cryo solution containing 0.1 M bis-Tris, pH 5.8, 2.1 M ammonium sulfate and 25% glycerol, and flash frozen in liquid nitrogen. The BimBH3 peptide sequence: DMRPEIWIAQELRRIGDEFNAYYAR. The SeMet-labeled protein was crystallized under the same conditions.

NMR Spectroscopy:

Data Collection: Native data was collected at ESRF ID14.1 (to 2.2 Å) and MAD data at ESRF ID 29 (to 2.5 Å).

Data Processing: All the datasets were processed with XDS. The peak and inflection point datasets were used to locate two Selenium sites with SOLVE. RESOLVE was used for density modification and subsequent model building. This initial model was used as a model in molecular replacement against the native data (MOLREP). The model was improved with ARP/WARP, that was able to build a 90% complete model. Manual model building was done with COOT and structure was refined with REFMAC5 using one TLS group covering all protein atoms. The structure was refined to R-factors R= 0.19 and Rfree= 0.25. Coordinates and structure factors for BCL2A1 were deposited to PDB with an accession code 2VM6.