

EBF3

PDB:3MUJ

Revision

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Entry Clone Accession:BC130479

Entry Clone Source:Mammalian Gene Collection

SGC Clone Accession:

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*smEATPCIKAIISPSEGWTTGGATVIIIGDNFFDGLQVVFGTMLVWSELITPHAIRVQTTPRHIPG
VVEVTL SYKSKQFCKGAPGRFVYTALNEPTIDYGFQRLQKVIPRHPGDPERLPKEVLLKRAADLVEALYGMPH

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 (32 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)

Porcedure

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 Ultra-15 centrifugal filter device, 5,000 NMWL (Millipore) to 2.7 mg/ml in a volume of 5 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 °C overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease on an Äktexpress system by passing the reaction mixture over a Ni-charged 1 ml HiTrap Chelating HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was retrieved from flow through as well as the wash with IMAC wash 2 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 5,000 MWCO (Sartorius). The final protein concentration was determined to 10.2 mg/ml in a volume of 0.5 ml. The result of the tag removal was confirmed by mass spectrometry.

Extraction

Procedure

The cell suspension was quickly 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 (49,000 x g, 20 min, 4 °C). The supernatant was decanted and filtered through a 0.45 µm flask filter.

Concentration:

Ligand

MassSpec:

Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl protein solution (10.2 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1 M bis-Tris propane pH 6.9, 0.2 M sodium formate and 21% PEG 3350. The plate was incubated at 4 °C and crystals appeared within 5 days. The crystals were quickly transferred to a cryo solution consisting of well solution complemented with 20% glycerol, and flash frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Diffraction data to 1.9 Å resolution was collected at BESSY BL14.1

Data Processing: The structure was solved by molecular replacement using TIG domain of EBF1 as a template (PDB: 3MQI). The space group was P65 with cell dimensions $a=134.68$ Å, $b=134.68$ Å, $c=41.15$ Å, $\alpha=90^\circ$, $\beta=90^\circ$, $\gamma=120^\circ$. Two monomers were located in the asymmetric unit. PHENIX was used for refinement and COOT for model building. Data in the interval 38.9-

1.9 Å resolution was used and at the end of the refinement the R values were: R=16.9% and R_{free}=19.3%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3MUJ.