EBF3

PDB:3N50

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:BC130479
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) 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:

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mhhhhhhssgvdlgtenlyfq*smSEATPCIKAISPSEGWTTGGATVIIIGDNFFDGLQVVFGTMLVWSELITPHAIRVQTPPRHIP
GVVEVTLSYKSKQFCKGAPGRFVYTALNEPTIDYGFQRLQKVIPRHPGDPERLPKEVLLKRAADLVEALYGMPHNNQEIILKRAADI
AEALYSV
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Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were plated on an LA plate with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Colonies from the plates were used to start a culture in 50 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (50 ml) was used to inoculate 4.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 1ml 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 (62 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 5 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 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.

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 room temperature for 1 h. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease on an ÄktaPurifyer system by passing the reaction mixture over a Ni-charged 1 ml HisTrap Chelating 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 19 mg/ml in a volume of 0.3 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 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the hagging drop vapour diffusion method in a 24-well plate. 2 µl protein solution (diluted to 10.7 mg/mlin GF buffer) was mixed with1 µl of well solution consisting of 0.1 M bis-Tris propane pH 7.5, and 2.9 M NaCl. The plate was incubated at 4 ºC and crystals appeared within14 days. The crystals were quickly transferred to a cryo solution consisting of 20% EG, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 3.1 Å resolution was collected at BESSY, beamline14-1
Data Processing: The structure was solved by molecular replacement using the EBF1 TIG domain (PDB: 3MQI) using PHASER. The space group was I 2 3 with cell dimensions a=b=c= 229.64 Å. Six monomers were located in the asymmetric unit. PHENIX was used for refinement and Coot for model building. Data in the interval 19.8 - 3.1 Å resolution was used and at the end of the refinement the R values were: R=18.83% and R_{free}=22.69%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3N50.