EBF1

PDB:3LYR

Revision

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Entry Clone Accession:BC038805
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:

mhhhhhhssgvdlgtenlyfq*smRSGSSMKEEPLGSGMNAVRTWMQGAGVLDANTAAQSGVGLARAHFEKQPPSNLRKSNFFHFVL
ALYDRQGQPVEIERTAFVGFVEKEKEANSEKTNNGIHYRLQLLYSNGIRTEQDFYVRLIDSMTKQAIVYEGQDKNPEMCRVLLTHEI
MCSRCCDKKSCGNRNETPSDPVIIDRFFLKFFLKCNQNCLKNAGNPRDMRRFQVVVSTTVNVDGHVLAVSDNMFVHNNSKHGRRARR
LDPS

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:SeMet labeled protein
Cells from a glycerolstock were plated on a LA 50 µg/ml kanamycin and 34 µg/ml chloramphenicol plate , colonies from the plate was used to inoculate the pre-culture in LB supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol, and grown at 30 °C overnight. 45 ml of the overnight culture were used to inoculate 3 bottles, each with 1.5 l minimal medium (without amino acids) supplemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and approximately 500 µl Dow Corning anti-foam RD emulsion 63213 4D (BDH Silicone Products) per bottle. The cultures were grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD_{600} reached ~0.5. The cultures were down-tempered to 18 °C and the amino acid mix was added. One hour later, expression of target protein was induced by addition of 0.5 mM IPTG. The expression was allowed to continue at 18 °C overnight and cells were harvested the following morning by centrifugation (4,500 x *g*, 10 min, 4 °C). The resulting cell pellet (21 g from 4.5 liter culture) was resuspended in lysis buffer (2ml/g cell pellet) supplemented with 1 tablet of Complete EDTA-free protease inhibitor (Roche Applied Science)

and 2000 U Benzonase (Merck) per 100 ml lysis buffer and stored at -80 °C.
Composition of 1 liter minimal media:1x M9 salts (Sigma), 0.4% (w/v) glucose, 2 mM $MgSO_4$, 0.1mM $ZnCl_2$, 250 µl trace elements (*Biotechnology and Bioengineering* **16**, 933-941 (1974)) and 0.5 mg thiamine. Mix of amino acids added per liter culture (Van Duyne, G. D., *J. Mol. Biol.* **229**, 105-124 (1993)): 100 mg each of lysine, threonine, phenylalanine and 50 mg each of leucine, isoleucine, valine, L(+)-selenomethionine.

Purification

Procedure
Columns
IMAC: Ni-charged 2x1 ml HiTrap Chelating HP (GE Healthcare) columns in seriesGel filtration column: HiLoad 16/60 Superdex 75 Prep Grade (GE Healthcare)Gel filtration column 2: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)

Procedure
At the day of purification the lysate was thawed in a water bath and filtered through a 0.45 µm flask filter. 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 columns and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC columns with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were pooled. The concentration was measured to 2.2 mg/ml in a volume of 13 ml and the sample was stored at -80 ºC. Full incorporation of selenomethionine was confirmed by mass spectrometry.

Tag removal
The protein was concentrad to 5 ml in Vivaspin 20 centrifugal filter device, 10,000 NMWL (Sartorius) followed by dilution to 20 ml with 30 mM HEPES, 10 % glycerol , pH 7.5 2 mM TCEP in order to reduce the NaCl level. The N-terminal histidine tag was proteolytically removed by incubating protein with His-tagged TEV protease at a molar ratio of 50:1. The proteolytic reaction went to completion, as judged by SDS-PAGE. The target protein was subsequently purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HisTrap HP column (GE Healthcare), pre-equilibrated with a buffer containing 20 mM HEPES, 500 mM NaCl, 10 % glycerol , pH 7.5 ,1 mM TCEP. The cleaved protein was eluted with addition of 15 mM imidazole to the buffer.The protein was concentrated and the buffer was changed to a buffer containing 300 mM NaCl, 20 mM HEPES, 10 % glycerol, 2 mM TCEP, pH 7.5 using a Vivaspin 20 centrifugal filter device, 10 000 NMWL (Sartorius). The final protein concentration was determined to 54 mg/ml in a volume of 0.75 ml.

DNA Complexation
The DNA oligomers 5´-GAGAGAGAGACTCAAGGGAATTGTGGCC and 5'-GGCCACAATTCCCTTGAGTCTCTCTCTC were annealed in a buffer composed of 10 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.5. Protein and double stranded DNA oligomer were mixed at a ration of 2.1: 1 and subjected to size exclusion chromatography using a HiLoad 16/60 Superdex 200 Prep Grade column (GE Healthcare) in a buffer containing 300 mM NaCl, 20 mM HEPES, 10 % glycerol, 2 mM TCEP, pH 7.5. The peak at a retention volume corresponding to a 2:1 complex was collected and the complex was concentrated in a Vivaspin 6 centrifugal filter device, 30 000 MWCO (Sartorius) to an approximate concentration of 0.5 mM.

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*, 30 min, 4 ºC). The supernatant was decanted and frozen in -80 ºC.
Concentration:
Ligand
MassSpec:
Crystallization: SeMet-labelled protein crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl of the protein and DNA complex solution (0.5 mM) was mixed with 0.1 µl of well solution consisting of 0.1 M MES pH 5.8, 2.1 M Ammonium Sulphate. The plate was incubated at 4 ºC and crystals were obtained after 5 days. The crystals were quickly transferred to cryo solution containing well solution and 20% glycerol, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Data was collected from a single crystal. Singl wavelenght SAD data was collected to 2.5 Å at DIAMOND (I02), Oxfordshire, England. The crystal belonged to space group P 65 2 2 with cell parameters of a=b=134.218 Å and c=72.391 Å.
Data Processing: The structure was solved using Shelx . Buccaneer was used to build the initial model, which was then improved by manual editing in COOT. The asymmetric unit consisted of a single monomer. The structure was refined with Refmac5. Final R-values were R= 23.3% and Rfree=27.4% and coordinates and structure factors were deposited in the PDB with accession code 3LYR.