

FKBP8

PDB:2AWG

Revision

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SGC Clone Accession:fkbp08.090.206:H3; SGC031 H3

Tag:His-tag with integrated thrombin-cleavage site MGSSHHHHHHSSGLVPR*GS.

Host:E.coliBL21 (DE3)

Construct

Prelude:

Sequence:

gsPEEWLDILGNLLRRKKTLPVGP PGSSRPVKGQVVTVHLQTSLENGTRVQEEPELVFTLGDCDVIQALDLSVPLMDVGETAMVTAD
SKYCYGPQGRSPYIPPHAALCLEVTLKTAVD

Vector:p28a-LIC

Growth

Medium:TB

Antibiotics:

Procedure:FKBP8 was expressed in E. coli BL21 (DE3) grown in Terrific Broth (TB) in the presence of 50 µg/ml of kanamycin at 37°C to an OD600 of 7.5. Cells were then induced by isopropyl-1-thio-D-galactopyranoside (IPTG), final concentration 0.05 mM, and incubated overnight at 15°C. The culture was centrifuged and the cell pellets were collected and stored at -80°C.

Purification

Procedure

IMAC purification: 4 microL of clarified supernatant is reserved for later analysis by SDS-PAGE. The rest of the clarified supernatant is then diluted 1:2 in lysis buffer, and loaded at approximately 1mL/min by gravity onto 5 mL of Ni-NTA resin (Qiagen 30450). 5 column volumes of lysis buffer are used to wash the column at approximately 3 mL/min, followed by 5 column volumes of low imidazole buffer (lysis buffer + 10 mM Imidazole (VWR EM-5720) pH 8) at approximately 3 mL/min. A 4 µL sample of the low imidazole wash is saved for later analysis by SDS-PAGE. Samples are eluted from the Ni-NTA resin by exposure to 10 mL elution buffer (lysis buffer + 250 mM imidazole and 10% glycerol (EMD GX0185-5)) at 1mL/min flow

rate. A 10 μ L sample of the eluate is saved for SDS-PAGE analysis. 10 μ L of each eluate is saved for measurement of protein concentration using Bradford reagent (BioRad 500-0202).

(His)6-tag cleavage: The 10 mL of Ni-NTA eluate from 2 is divided into 2x5 mL samples in 50 mL conical vials (352096, BD Biosciences). 5 mL of uncut sample is loaded onto gel filtration (see below); 1 unit of thrombin (Sigma T9681) per milligram of protein is added to the other 5 mL sample. The conical vial is stored without shaking, overnight, at 4°C.

Size exclusion chromatography: All gel filtration columns, buffers, and protocols are identical for uncut and thrombin-treated proteins. A XK 16x65 column (part numbers 18-1031-47 and 18-6488-01, GE Healthcare) packed with HighLoad Superdex 200 resin (10-1043-04, GE Healthcare) is pre-equilibrated with gel filtration buffer (lysis buffer + 5 mM β -ME (Sigma 63689)) for 1.5 column volumes using an AKTApurifier (18-6645-05, GE Healthcare) at a flow rate of 3 mL/min. 5 mL of sample is loaded onto the column at 1.5 mL/min, and 2mL fractions are collected into 96-well plates (VWR 40002-012) using peak fractionation protocols with the following parameters: (Slope; min. peak width 0.833 min; level 0.000 mAU; peak start slope 10.000 AU/min; peak end slope 20.000 AU/min). Peak fractions are analyzed for purity using SDS-PAGE or visual analysis of the chromatogram and pooled.

Concentration: Purified proteins are concentrated using either 4 mL or 15 mL concentrators with an appropriate molecular weight cut-off (Amicon Ultra-15 10,000 MWCO, UFC901024 or 5,000 MWCO, UFC900524, as appropriate, Millipore) to a final concentration of 20 mg/mL for crystallographic screening or other biophysical studies.

Extraction

Procedure

Frozen cell pellets contained in bags (Beckman 369256) obtained from 2L liters of culture are thawed by soaking in warm water for 5 minutes. Each cell pellet is resuspended in 20 mL lysis buffer + 1mM phenylmethanesulfonyl fluoride (Sigma P7626), and 1mL Sigma general protease inhibitor (Sigma P2714-1BTL), resuspended according to manufacturer's instructions) and then homogenized using an Ultra-Turrax T8 homogenizer (IKA Works) at maximal setting for 30-60 seconds per pellet. Cell lysis is accomplished by sonication (Virtis408912, Virsonic) on ice: the sonication protocol is 10 sec pulse at half-maximal frequency (5.0), 10 second rest, for 6 minutes total sonication time per pellet. Lysed cells are placed into centrifuge tubes (363647, Beckman Coulter) and centrifuged in a JA25.50 rotor in an Avanti J-20 XPI centrifuge (Beckman Coulter) for 20 minutes at 69,673 x g. The supernatant is decanted into a beaker, and the insoluble pellet discarded.

Concentration:

Ligand

MassSpec:

Crystallization: Purified FKBP8 was crystallized using the hanging drop vapor diffusion method. Crystals grew when the protein was mixed with the reservoir solution in a 1:1 volume ratio, and the drop was equilibrated against a reservoir solution containing 0.5M NH_4SO_4 , 1M LiSO_4 , 0.1M Na-Citrate at pH 5.6 in 293K temperature, with 15% glycerol added as cryo protectant.

NMR Spectroscopy:

Data Collection:

Data Processing: