BAK1A

PDB:2JCN

Revision

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Revised by:created
Revision Date:created
Entry Clone Accession:BC004431
Entry Clone Source:MGC
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*s(m).
Host:BL21 (DE3)

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqSMSASEEQVAQDTEEVFRSYVFYRHQQEQEAEGVAAPADPEMVTLPLQPSSTMGQVGRQLAIIGDD
INRRYDSEFQTMLQHLQPTAENAYEYFTKIATSLFESGINWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWI
AQRGGWVAALNLGNGPILN

Vector:pNIC28-Bsa4

Growth

Medium:TB, supplemented with 8 g/L glycerol
Antibiotics:
Procedure: Cells (BL21(DE3)) from frozen glycerol stocks were grown in 2x20 mL of Terrific Broth, supplemented with 8 g/L glycerol, 100 µg/mL kanamycin, at 37°C over night. The following morning, 2x20 mL of the over night cultures inoculated 2x1500 mL Terrific Broth with 8 g/L glycerol , 50 µg/mL kanamycin, and 100 µL BREOX (antifoam). Cultivation was performed in glass flasks in the Large Scale Expression System (LEX). Cells were grown at 37°C until an OD600 nm of 2 was reached. The cultivations were down-tempered to 18 °C for 1h. Expression of target protein was induced by addition of 0.5 mM IPTG and was allowed to continue over night at 18 °C.

Purification

Procedure
Columns:
HiTrap Chelating 1 mL (IMAC) and HiTrap IMAC 1mL
HiLoadÂ□ 16/60 Superdex 75 Prep Grade (Gel filtration).

The purification was conductive manually using a peristatic pump. The duplicates were mixed and divided into two so the same amount of lysate was loaded onto the two columns (HiTrap Chelating 1mL and HiTrap IMAC 1mL). An affinity blank run was first done on the columns to avoid Ni-leakage (5 CV H2O, 5 CV W1, 5 CV E, 10 CV W1). The sample was loaded onto the columns and washed with Wash 1 buffer (5-10 CV), and Wash 2 buffer (20 CV), and eluted in elution buffer (about 18 CV). Flowrate during the runs, 1 mL/min. The protein was collected in 2 mL fractions and was detected and evaluated on SDS-PAGE. The samples were pooled and loaded onto the Gel filtration column, 16/60 Superdex 75 Prep Grade. The gelfiltration chromatogram showed one major protein peak that mainly consisted of BAK1A-c005 as shown by SDS-PAGE analysis. The protein was TEV cleaved for 4 hours and the sample was then run over a Ni-HisTrap column. The flowthrough was concentrated to 40.6 mg/mL in 0.7 mL, stored at -80ºC.

Extraction

Procedure
Cells were harvested by centrifugation (10 min, 4400 ×g) and pellets were resuspended in 50mM Sodium-Phosphate pH 7.5, 500mM NaCl, 10% glycerol, 0.5 mM TCEP (IMAC Bind/Wash1 Buffer) supplemented with one tablet of Complete EDTA-free protease inhibitor tablet and freezed at -80 ºC. The cells were quickly thawed in warm water and 4ul (1000 U) of Benzonase was added. Cells were disrupted by sonication (4s on 4s off 3 min 80% amplitude) and the lysate was centrifuged for 20 minutes at 49 000×g. The supernatant was decanted and filtered through 0.45µm filter prior to loading onto the columns, HiTrap Chelating 1mL, HiTrap IMAC 1mL and Sephadex75 for furher purification.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals was obtained by mixing 0.1+0.1 microL drops of protein solution with a well solution containing 20% (w/v) peg3350 and 0.2M sodium sulfate and were then frozen using a cryo solution containing 22% (w/v) peg3350, 20% glycerol, 0.2M sodium sulfate, 2mM TCEP, 0.3M sodium chloride, and 20mM HEPES pH 7.5. Derivative crystals were obtained by soaking experiments using saturated amounts of KAu(CN)2 and K2Cl4Pt in cryo solution for 10 min prior to freezing.
NMR Spectroscopy:
Data Collection:
Data Processing: The datasets were processed in XDS and scaled individually scaled in XSCALE before submission to XPREP for further scaling against each other. An anomalous signal was seen for both derivatives however the signal were stronger for Pt than for Au. The native data and the two derivatives were then submitted as a MIRAS job for SOLVE and RESOLVE that automatically solved and built a poly-glycine model of BAK1 with several helices. This crude model was then submitted to ARP/WARP that managed to complete 90% of all atoms using the 1.8Å dataset only. Final cycles of model building were performed in COOT using REFMAC as the refinement program.