ITPKC

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Revision

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Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*s(m).
Host:E. coli Rosetta2(DE3)-pLysS

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqsMPWVQLSGHAGNFQAGEDGRILKRFCQCEQRSLEQLMKDPLRPFVPAYYGMVLQDGQTFNQMEDL
LADFEGPSIMDCKMGSRTYLEEELVKARERPRPRKDMYEKMVAVDPGAPTPEEHAQGAVTKPRYMQWRETMSSTSTLGFRIEGIKKA
DGTCNTNFKKTQALEQVTKVLEDFVDGDHVILQKYVACLEELREALEISPFFKTHEVVGSSLLFVHDHTGLAKVWMIDFGKTVALPD
HQTLSHRLPWAEGNREDGYLWGLDNMICLLQGLAQS

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure:10 µL competent Rosetta2-pLysS cells (Novagen) were transformed with 1 µL plasmid. Held 30min on ice, heatshocked in 42 degree waterbath for 45sec at 42°C. Held on ice for 2 min. Added 100µL SOC and incubated in shaker for 1 h. Cells were plated on LA plates with 50 mg/l Kanamycin, 34 mg/l Chloramphenicol and 0.2% glucose. Colonies were grown in 130 mL of Terrific Broth (TB) with 50 mg/l Kanamycin and 34 mg/l Chloramphenicol at 30°C overnight. 10 ml of overnight culture was added to a TunAir flask with 1 liter of TB with 50 mg/l Kanamycin and 34 mg/l Chloramphenicol. The culture was incubated at 37°C, until OD600 reached of approximately 1.4. The temperature was lowered to 25°C and the culture was induced with 0.5 mM IPTG for 4 hours.

Purification

Procedure
Column:

1.3 ml of Nickel charged Metal Chelating Fast Flow resin slurry (Amersham Biosciences) added to an Econo-Pac gravity flow column (Biorad).

Purifications were carried out at +4°C. After sample loading, the IMAC gravity flow column was washed with 10 ml of IMAC Bind/Wash1 buffer followed by 10 ml of IMAC Wash2 buffer. Elution was performed by addition of IMAC elution buffer in five 1ml fractions. Elution fractions were pooled based on SDS-PAGE analysis. Protein was estimated by SDS-PAGE analysis to be more than 95% pure. TCEP was added to the pooled protein to a final concentration of 2 mM. Using a 10 ml solvent absorption device (Vivapore) with 7500 Da cutoff, the protein was concentrated to 20 mg/mL coupled with a simultaneous buffer exchange to 20 mM Tris pH 8.0, 150 mM NaCl, 10 % glycerol and 5 mM TCEP. Yield of purified protein per liter of culture was 13 mg.

Extraction

Procedure
Cells were harvested by centrifugation (total of 18 g cells) and pellets were resuspended in 50mM HEPES pH 7.5, 500mM NaCl, 10% glycerol, 0.5 mm TCEP and 1 tablet Complete EDTA-free protease inhibitor (Roche Biosciences). Cells were disrupted by one pass at 15000 psi in a high-pressure homogenizer (Stansted). DNA precipitation was performed by addition of PEI to a final concentration of 0.15%. The sample was incubated on ice for 30 minutes and centrifuged for 20 minutes at 20000×g. The soluble fraction was filtered through 0.22 µm and subjected to further purification.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained using sitting drop method at 20°C. Drops were prepared using 1 µl of protein (10 mg/ml concentration with 1 mM $MgCl_2$, 1 mM AMP-PNP and 1 mM D-myo-Inositol-1,4,5-triphosphate) and 1 µl of the well solution (40% Ethylene glycol, 0.1 M Acetate buffer pH 5.0). Boat-shaped crystals grew overnight.
NMR Spectroscopy:
Data Collection:
Data Processing: