Cp-CDPK4

PDB:2WEI

Revision

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Entry Clone Accession:cgd3_920
Entry Clone Source:
SGC Clone Accession:cgd3_920:T71-E338:E7
Tag:mhhhhhhssgrenlyfqg
Host:BL21(DE3)R3pACYC-LIC+LamP-phosphatase

Construct

Prelude:
Sequence:

mhhhhhhssgrenlyfqgTFAERYNIVCMLGKGSFGEVLKCKDRITQQEYAVKVINKASAKNKDTSTILREVELLKKLDHPNIMKLF
EILEDSSSFYIVGELYTGGELFDEIIKRKRFSEHDAARIIKQVFSGITYMHKHNIVHRDLKPENILLESKEKDCDIKIIDFGLSTCF
QQNTKMKDRIGTAYYIAPEVLRGTYDEKCDVWSAGVILYILLSGTPPFYGKNEYDILKRVETGKYAFDLPQWRTISDDAKDLIRKML
TFHPSLRITATQCLEHPWIQKYSSE

Vector:p15-mhl

Growth

Medium:TB
Antibiotics:
Procedure: The protein was expressed in E. coli BL21(DE3)R3pACYC-LIC+LamP-phosphatase cells in Terrific Broth (TB) in the presence of ampicillin/chloramphenicol (50 microg/mL and 25 microg/mL respectively). A single colony was inoculated into 10 mL of LB with of ampicillin/chloramphenicol (50 microg/mL and 25 microg/mL respectively) in a 50 mL Falcon tube and incubated with shaking at 250 rpm overnight at 37 degC. The culture was transferred into 50 mL of TB with 50 microg/mL ampicillin in a 250 mL shaking flask and incubated at 37 degC for 3 hours. Then the culture was transfered into 1.8 L of TB with 50 microg/mL kanamycin

and 0.3 mL of antifoam (Sigma) in a 2 L bottle and cultured using the LEX system to an OD 600 of ~5, cooled to 15 degC, and induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) overnight at 15 degC.

Purification

Procedure
STEP1:The cleared lysate was loaded onto a column prepacked with 10 g DE52 (Whatman) anion exchange resin (previously activated with 2.5 M NaCl and equilibrated with Binding Buffer); and subsequently onto a 3 mL Ni-NTA (Qiagen) column pre-equilibrated with Binding Buffer at approximately 1 - 1.5 mL/min. The volume of the Ni-NTA resin was pre-determined by the predicted protein yield from test expression analysis. After the lysate was loaded, the DE52 was further washed with 20 mL of Binding Buffer. The Ni-NTA column was then washed with 200 mL of Wash Buffer at 2 - 2.5 mL/min. After washing, the protein was eluted with 15 mL of Elution Buffer. EDTA was immediately added to the elution fraction to 1 mM; and TCEP was added to 0.5 mM after approximately 15 more minutes.

STEP2:The sample was loaded onto a Sephadex S200 26/60 gel filtration column pre-equilibrated with 10 mM HEPES, pH 7.5 and 500 mM NaCl, 5mMDTT. The collected fractions corresponding to the correct eluted protein peak were concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). The protein sample identity were evalulated by mass spectroscopy. The concentrated sample (58.6 mg/ml) was stored at 4 degC.

Extraction

Procedure
The culture was harvested by centrifugation. Pellets from 4 L of culture were resuspended to approximately 40 mL/L of cell culture in Binding Buffer with the addition of protease inhibitors (1 mM benzamidine and 1 mM phenylmethyl sulfonyl fluoride (PMSF)). Resuspended pellets stored at -80 degC were thawed overnight at 4 degC on the day before purification. Prior to mechanical lysis, each pellet from 1 L of culture was pretreated with 0.5 % CHAPS and 500 units of benzonase for 40 minutes at room temperature. Cells were mechanically lysed with a microfluidizer (Microfluidizer Processor, M-110EH) at approximately 18000 psi; and the cell lysate was centrifuged using a Beckman JA-25.50 rotor at ~75000 x g (24000 rpms) for 20 minutes at 10 degC.
Concentration:
Ligand
3MB-PP1**MassSpec:**
Crystallization: The protein sample precipitated slightly after thawing and was therefore centrifuged and the concentration remeasured to 26 g/L prior to being mixed with 0.5 mM of 3MB-PP1. After 15 min incubation at room temperature and a second brief centrifugation crystallisation experiments were set up in sitting drops of 50 nL protein and 100 nL well solution at 20 degC. Crystals of a prism shaped morphology appeared in well H3 of the JCSG+ coarse

screen, 25% PEG 3350 and 0.1M BIS-TRIS pH 5.5, during the first 24 hours and grew to a final size of ~100 microns in 3 days. For cryo protection the crystals were transferred to a drop of well solution supplemented with 25% glycerol (v/v) before flash cooling in liquid nitrogen. Crystal manipulation was performed in a cold room at 4 degC.

NMR Spectroscopy:

Data Collection:

Data Processing: Data were collected from a single crystal at beam line X10SA at the Swiss Light Source. Processing was performed in Mosflm and Scala to a resolution of 1.65 in space group P43212, with the cell 68.90, 68.90, 130.46, 90.00, 90.00, 90.00. A molecular replacement solution was easily found with Phaser using the apo structure's coordinates (PDB code 3DFA) as the search model. Refinement in REFMAC5 resulted in the final deposited model with an R=19.7 and Rfree=22.9%.