cgd7_1840

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Revision

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Entry Clone Accession:cgd7_1840
Entry Clone Source:
SGC Clone Accession:cgd7_1840:S197-L472; plate MAC024:D9
Tag:N-terminal His6-tag with integrated TEV cleavage site (*): mhhhhhhssgrenlyfq*g
Host:*E. coli* BL21-(DE3)-R3-pRARE2

Construct

Prelude:
Sequence:

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mhhhhhhssgrenlyfqgSTKGDINQYYTLENTIGRGSWGEVKIAVQKGTRIRRAAKKIPKYFVEDVDRFKQEIEIMKSLDHPNIIR
LYETFEDNTDIYLVMELCTGGELFERVVHKRVFRESDAARIMKDVLSAVAYCHKLNVAHRDLKPENFLFLTDSPDSPLKLIDFGLAA
RFKPGKMMRTKVGTPYYVSPQVLEGLYGPECDEWSAGVMMYVLLCGYPPFSAPTDSEVMLKIREGTFTFPEKDWLNVSPQAESLIRR
LLTKSPKQRITSLQALEHEWFEKQLSSSPRNLL
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Vector:p15-mhl

Growth

Medium:TB
Antibiotics:100 microG/mL ampicillin and 34 microG/mL chloramphenicol
Procedure:A single colony was inoculated into 10 mL of LB with of Antibiotics and incubated with shaking at 250 rpm overnight at 37 °C. The culture was transferred into 50 mL of TB with Antibiotics in a 250 mL shaking flask and incubated at 37 °C for 3 hours. The culture was then transferred into 1.8 L of above-specified growth medium with Antibiotics and 0.3 mL of antifoam (Sigma) in a 2L bottle and cultured using the LEX system to an OD600 of ~5, cooled to 15 °C and induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) overnight at 15 °C.

Purification

Procedure
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. MgCl2 and CaCl2 was added to the elution fraction to 1 mM; and TCEP was added to 2 mM after elutting from Ni-NTA column.

The sample was loaded onto a Sephadex S200 26/60 gel filtration column pre-equilibrated with Gel Filtration Buffer. The collected fractions corresponding to the correct eluted protein peak were concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). TCEP (2mM), 1mM $MgCl_2$, 2mM $CaCl_2$ was added to the concentrated protein. The protein sample identity were evalulated by mass spectroscopyand found to be a mixture of different phosphorylated states.The protein was treated by lambda protein phosphatase from Biolabs.Subsequent evaluation by mass spectroscopy again revealed thatthe phosphatase resulted in a dominant species with a single phosphorylation site.

Extraction

Procedure
Cells were resuspended to approximately 40 mL/L of cell culture in Binding Buffer with protease inhibitor (1 mM benzamidine-HCl and 1 mM phenylmethyl sulfonyl fluoride, PMSF). Resuspended pellets stored at -80 ºC were thawed overnight at 4 ºC 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 at ~75000 x g for 20 minutes at 10 ºC.
Concentration: The concentrated sample (20 mg/mL) was stored at 4 °C.
Ligand
MassSpec:
Crystallization: The protein was crystallized using the hanging drop vapor diffusion method at 20 ºC in 18% PEG 3350, 0.1 M NH_4SO_4, 0.1 M NaCacodylate, 5 mM Indirubin E804, pH 5.5.
NMR Spectroscopy:
Data Collection:
Data Processing: