PIM1 phosphorylated

PDB:2BIK

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession: The expressed protein has the sequence from gi 33304198 which differs from gi 4505811 by a single change R250G (red in the sequence below)
Entry Clone Source:TSK
SGC Clone Accession:
Tag:N-terminal His-tag with integrated TEV protease site:
MHHHHHHSSGVDLGTENLYFQ*SH
Host:BL21(DE3)

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqsMLLSKINSLAHLRAAPCNDLHATKLAPGKEKEPLESQYQVGPLLGSGGFGSVYSGIRVSDNLPVA
IKHVEKDRISDWGELPNGTRVPMEVVLLKKVSSGFSGVIRLLDWFERPDSFVLILERPEPVQDLFDFITERGALQEELARSFFWQVL
EAVRHCHNCGVLHRDIKDENILIDLNRGELKLIDFGSGALLKDTVYTDFDGTRVYSPPEWIRYHRYHGRSAAVWSLGILLYDMVCGD
IPFEHDEEIIGGQVFFRQRVSpSECQHLIRWCLALRPSDRPTFEEIQ NHPWMQDVLLPQETAEIHLHSLSPGPS

Vector:pLIC-SGC

Growth

Medium:
Antibiotics:
Procedure:10 ml overnight cultures in LB, 100 µg/ml ampicillin were centrifuged, resuspended in fresh buffer, and used to inoculate 1 litre of LB medium containing 100 µg/ml ampicillin. Cultures were grown at 37oC until they reached an OD600 of 0.3 and then cooled to 18oC. Expression was induced for 4 hours using 1 mM IPTG at an OD600 of 0.8. The cells were collected by centrifugation, transferred to 50 ml tubes, resuspended in 30 ml binding buffer, and frozen. Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol.

Purification

Procedure
Column 1: Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatmann), 10 g of resin in 2.5 x 20 cm column. The resin was hydrated in 2.5M NaCl, then washed with 20 ml binding buffer prior to loading the sample.

Buffers: Binding buffer

Procedure: Supernatant was applied at gravity flow, followed by a wash with 20 ml binding buffer. The column flow-through was collected.

Column 2: Ni-affinity. Ni-NTA (Qiagen), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Buffers: Binding buffer:50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol. Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 20 mM Imidazole, 5% glycerol. Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole, 5% Glycerol.

Procedure: The flowthrough from column 1 was loaded by gravity flow on the Ni-NTA column. The column was then washed with 3 x 10 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 250 mM); fractions were collected until essentially all protein was eluted. After elution DTT was added to a final concentration of 10 mM.

Column 3: Ion exchange Mono Q column.

Buffers: A :50 mM Hepes pH 7.5>. B : 50 mM Hepes pH 7.5, 1000 mM NaCl

Procedure: Partially dephosphorylated Pim1 was applied to MonoQ in buffer A and eluted from the column by a linear gradient.

Concentration: Pim1 samples containing mono phosphorylated protein were pooled and concentrated in Centricons (10 kDa cut off). Phosphorylation was monitored using LC-ESI MS-Tof.

Enzymatic treatment: Dephosphorylation (l-phosphatase): a GST fusion with the lambda phosphatase. TEV protease cleavage. Both treatments carried out simultaneously: protein solution contained 10 mM DTT and 0.05 mM MnCl2 (higher MnCl2 concentrations caused precipitation).

The purified protein was homogeneous and had an experimental mass of 35626 Da as expected from its primary structure plus single phosphorylation.

Extraction

Procedure
The frozen cells were thawed on ice and lysed using a high pressure cell disruptor. The lysate was centrifuged at 17,000 RPM for 30 minutes. Supernatant was collected and binding buffer was added to 50 ml.
Concentration:
Ligand
MassSpec:
Crystallization: The BIM-1 inhibitor (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide, HCl) was purchased from Calbiochem and added at a concentration of 1 mM from a 50 mM DMSO stock solution to the protein. Crystals were grown at 4o C in 3 µl sitting drops mixing 1.5 µl Pim1 (10 mg/mL in 50mM Hepes pH 7.5, 280mM NaCl, 5% glycerol ,10mM DTT with 1.5 µl of a solution containing 0.2 M Na2SO4, 20% PEG 3350, 10% ethylene glycol and 0.5% DMSO.
NMR Spectroscopy:

Data Collection:
Data Processing: