PIM1 with Peptide and BIM-1

PDB:2BIL

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
IPFEHDEEIIGGQVFFRQRVSSECQHLIRWCLALRPSDRPTFEEIQ 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
Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatmann), 10 gr 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. Supernatant was applied at gravity flow, followed by a wash with 20 ml binding buffer. The column flow-through was collected.

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

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. 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.

Concentration: Samples containing Pim1 were pooled and alkaline phosphatase was added in the same buffer. Protein sample was treated for 12 h.

Ion exchange Mono Q column: Buffers: A : 50 mM Hepes pH 7.5. B : 50 mM Hepes pH 7.5, 1000 mM NaCl. Dephosphorylated Pim1 was applied to MonoQ in buffer A and eluted from the column by a linear gradient. Pim1 samples containing unphosphorylated protein were pooled and concentrated in Centricons (10 kDa cut off). Dephosphorylation was monitored using LC-ESI MS-Tof. 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).

Masses of purified proteins were confirmed by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% acetonitrile in water with 0.1% formic acid.

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. Consensus peptide (Ala-Arg-Arg-Arg-His-Pro-Ser) was added to approximately 1.6 times excess of the protein concentration (i.e. 450µM peptide compared to 280µM protein). Crystals were grown at 4oC 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: