PTK2B

PDB:3CC6

Revision

Revision Type:created
Revised by:created
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Entry Clone Accession:gi|27503699
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21-Gold (DE3) (Stratagene) also harboring a plasmid for co-expression of YopH phosphatase (Seeliger, M.A., High yield bacterial expression of active c-Abl and c-Src tyrosine kinases.*Protein Sci.* **14**, 3135-3139 (2005)).

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*sMGGPQYGIAREDVVLNRILGEGFFGEVYEGVYTNHKGEKINVAVKTCKKDCTLDNKEKFMSEAV
IMKNLDHPHIVKLIGIIEEEPTWIIMELYPYGELGHYLERNKNSLKVLTLVLYSLQICKAMAYLESINCVHRDIAVRNILVASPECV
KLGDFGLSRYIEDEDYYKASVTRLPIKWMSPESINFRRFTTASDVWMFAVCMWEILSFGKQPFFWLENKDVIGVLEKGDRLPKPDLC
PPVLYTLMTRCWDYDPSDRPRFTELVCSLSDVYQMEKDIAME

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 40 ml LB supplemented with 50 µg/ml kanamycin, 50 µg/ml streptomycin and 50 µl BREOX FMT 30 anti-foam solution (Cognis Performance Chemicals UK Ltd) at 37 ºC overnight. 30 ml of the overnight culture were used to inoculate 4 x 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin. The culture was grown in TunAir flasks at 37 ºC until OD600 reached ~1. The cultures were down-tempered to 18 ºC over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 ºC). The resulting cell pellet (84.7 g wet cell weight) was stored at -80 ºC.

Purification

Procedure

Columns
IMAC: 3 ml Ni-NTA Agars in a CellThru 10 ml disposable column (CloneTech)
Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)
IEX column: Mono Q 5/50 GL (GE Healthcare)

Procedure
Purification of the protein was performed as a three step process by gravity flow and an ÄKTAprime system (GE Healthcare). Prior to purification, columns were equilibrated with lysis buffer, gel filtration buffer and Mono Q A buffer, respectively. The filtered lysate was loaded onto the Ni-NTA sepharose column and washed with IMAC wash1 buffer. Bound protein was eluted from the IMAC column with 3 x 5 ml IMAC elution buffer. The first eluted sample was, after addition of 2 mM TCEP, concentrated to 2.5 ml, filtered through 0.22 µm and loaded onto the gel filtration column. Fractions containing the target protein were pooled and the protein was subsequently concentrated in an Amicon Ultra-15 centrifugal filter device with 10,000 NMWL (Millipore) to a volume of 0.5 ml. In the last step the protein was loaded onto a Mono Q ion exchange column and washed with Mono Q buffer A before eluting the protein in a gradient of Mono Q buffer B. Selected fractions were pooled and fresh TCEP was added to 2 mM. The protein was concentrated to 200 µl, diluted in 20 ml gel filtration buffer and re-concentrated in an Amicon Ultra-15 centrifugal filter device with 10,000 NMWL (Millipore)to 24.1 mg/ml in a volume of 0.41 ml.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease (van den Berg, S., *J. Biotech* **121**, 291-298 (2006)) at a w/w ratio of 20:1 at 4 ºC for 48 hours. The proteolytic reaction went to completion, as judged by SDS-PAGE. 10 mM imidazole was added and the target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HisTrap HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The cleaved protein was concentrated and the buffer was changed to GF buffer containing 2 mM TCEP using an Amicon Ultra-15 centrifugal filter device with 10,000 NMWL (Millipore). The final protein concentration was determined to 24.6 mg/ml in a volume of 0.15 ml. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell pellet was thawed and resuspended in lysis buffer (2 ml/g cell pellet), supplemented with 3000 U Benzonase (Merck) and 1.5 tablets of Complete EDTA-free protease inhibitor (Roche Applied Science) and a knife edge of lysozyme (Sigma). Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl of the protein sample (diluted to 15 mg/ml) including 50 nM S Taurosporin was mixed with 0.2 µl of well solution consisting of 0.1 M Bis-Tris pH 5.5, 0.05 M MgCl2, 17.5% PEG 3350. The plate was incubated at 4 ºC and rod shaped crystals appeared within 3 days. The crystals were quickly transferred to cryo solution containing 0.1 M Bis-Tris pH 5.5, 0.1 M MgCl2, 25% PEG 3350, 0.3 M NaCl and 25% glycerol and flash-frozen in liquid nitrogen.

NMR Spectroscopy:

Data Collection: Data to 1.6 Å resolution was collected at ESRF beamline ID29. A single crystal was used to collect 360º degrees oscillation range.

Data Processing: Data was processed with XDS and solved by MR using Molrep. The space group was P21 with cell dimensions a=37.45 Å, b=96.14 Å, c=43.20 Å, α=90.00, β=93.66, γ=90.00. Automatic model building using Arp/warp was performed; Refmac5 was used for refinement and Coot for model building. Data in the interval 20.0-1.60 Å resolution was used during refinement. Final values for R and Rfree were 18.4% and 21.9%, respectively. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3CC6.