HIBCH

PDB:3BPT

Revision

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Entry Clone Accession:IMAGE:5266813
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SGC Clone Accession:
Tag:C-terminal, TEV cleavable hexahistidine tag. Tag sequence: aenlyfq(*)shhhhhhdykddddk
Host:E. coli B834 (DE3) for SeMet labelling

Construct

Prelude:
Sequence:

MTDAAEEVLLGKKGCTGVITLNRPKFLNALTLNMIRQIYPQLKKWEQDPETFLIIIKGAGGKAFCAGGDIRVISEAEKAKQKIAPVF
FREEYMLNNAVGSCQKPYVALIHGITMGGGVGLSVHGQFRVATEKCLFAMPETAIGLFPDVGGGYFLPRLQGKLGYFLALTGFRLKG
RDVYRAGIATHFVDSEKLAMLEEDLLALKSPSKENIASVLENYHTESKIDRDKSFILEEHMDKINSCFSANTVEEIIENLQQDGSSF
ALEQLKVINKMSPTSLKITLRQLMEGSSKTLQEVLTMEYRLSQACMRGHDFHEGVRAVLIDKDQSPKWKPADLKEVTEEDLNNHFKS
LGSSDLKFaenlyfq

aenlyfq residues originate from the vector and remain after the TEV cleavage of the hexahistidine tag.

Vector:pNIC-CTHF

Growth

Medium:
Antibiotics:
Procedure:A single colony of B834 (DE3) was added to 200ml of LB with 50µg/ml kanamycin and grown at 30°C overnight. Harvested cells were washed twice with PBS and resuspended in MD medium. 10ml were inoculated into 1L of MD medium with 50µg/ml kanamycin and 40mg of Selenomethionine powder (8L in total) Cultures were grown at 37°C. At the OD of 1 the temperature was lowered to 25°C and an hour later 0.5mM IPTG was added. The next day cells were harvested by centrifugation at 4000 rpm for 10min and stored at -20°C.

Purification

Procedure

Purification: Column 1: Ni-affinity, His-Trap, 1ml (Amersham); Column 2: Superdex 200, HiPrep 16/60 (Amersham)

Buffers: Start buffer: 50mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP; Washing buffer: 50mM HEPES pH 7.5, 500mM NaCl, 40mM Imidazole, 5% glycerol, 1mM PMSF, 0.5mM TCEP; Elution buffer: 50mM HEPES pH 7.5, 500mM NaCl, 5% glycerol, 250mM Imidazole, 0.5mM TCEP.

GF buffer: 50mM HEPES pH 7.5, 150mM NaCl, 0.5mM TCEP

Procedure: The cell extract was loaded on an AKTA Express system. The extinction at 280nm was monitored and fractions were collected and analyzed by SDS-PAGE. Positive fractions were pooled for TEV cleavage. A single resulting peak was characterised by mass spectrometry.

TEV cleavage: The His-tag was cleaved with 1mg TEV per 40mg target protein at 4°C overnight.

Extraction

Procedure
Lysis buffer: 50mM HEPES pH 7.5, 500mM NaCl, 20mM Imidazole, 5% glycerol + EDTA-free Complete (1 tablet/50ml). The thawed cells were resuspended in the lysis buffer and broken by 5 passes at 16.000 psi through a high pressure homogeniser followed by centrifugation for 45min at 15.000rpm.
Concentration: Concentration and buffer exchange: Using Amicon Ultra-15 concentrators with 30 kDa cutoff, the sample was concentrated to 65mg/ml. Concentrations were determined from the absorbance at 280 nm using NanoDrop. Electron density has shown the N-terminal SeMet to be present.
Ligand
quercetin**MassSpec:**The calculated mass of the construct was 41026. The exact mass was confirmed by mass spectrometry, together with a second peak arising from the lack of the N-terminal SeMet (40848).
Crystallization: Crystals were grown by vapor diffusion at 20°C in 320nl sitting drops. Quercetin was added to a final concentration of 5mM prior to crystallisation. The drops were prepared by mixing 100nl of protein solution and 200nl of precipitant consisting of 0.1M citrate pH 5.5 and 20% PEG 3350. Then the drops were seeded with 20ul of suspended micro-seeds created from the native crystals. Crystals were transferred to a cryo-protectant consisting of the well solution and 20% PEG 400 before flash-cooling in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Resolution: 1.7Å; X-ray source: SLS beam X10SA.
Data Processing: