Entry Clone Source: Ordered-Geneservice
Entry Clone Accession: IMAGE:8143970
SGC Construct ID: THB-c106
GenBank GI number: gi|88900503
Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

CTTAAGAAGGAGATATACTATGGTCC
CCTGGTTCCCAAGAAAAGTGTCAGAG
CTGGACAAGTGTCATCACCTGGTCAC
CAAGTTCGACCCTGACCTGGACTTGG
ACCACCCGGGCTTCTCGGACCAGGTG
TACCGCCAGCGCAGGAAGCTGATTGC
TGAGATCGCCTTCCAGTACAGGCACG
GCGACCCGATTCCCCGTGTGGAGTAC
ACCGCCGAGGAGATTGCCACCTGGAA
GGAGGTCTACACCACGCTGAAGGGCC
TCTACGCCACGCACGCCTGCGGGGAG
CACCTGGAGGCCTTTGCTTTGCTGGA
GCGCTTCAGCGGCTACCGGGAAGACA
ATATCCCCCAGCTGGAGGACGTCTCC
CGCTTCCTGAAGGAGCGCACGGGCTT
CCAGCTGCGGCCTGTGGCCGGCCTGC
TGTCCGCCCGGGACTTCCTGGCCAGC
CTGGCCTTCCGCGTGTTCCAGTGCAC
CCAGTATATCCGCCACGCGTCCTCGC
CCATGCACTCCCCTGAGCCGGACTGC
TGCCACGAGCTGCTGGGGCACGTGCC
CATGCTGGCCGACCGCACCTTCGCGC
AGTTCTCGCAGGACATTGGCCTGGCG
TCCCTGGGGGCCTCGGATGAGGAAAT
TGAGAAGCTGTCCACGCTGTACTGGT
TCACGGTGGAGTTCGGGCTGTGTAAG
CAGAACGGGGAGGTGAAGGCCTATGG
TGCCGGGCTGCTGTCCTCCTACGGGG
AGCTCCTGCACTGCCTGTCTGAGGAG
CCTGAGATTCGGGCCTTCGACCCTGA
GGCTGCGGCCGTGCAGCCCTACCAAG
ACCAGACGTACCAGTCAGTCTACTTC
GTGTCTGAGAGCTTCAGTGACGCCAA
GGACAAGCTCAGGAGCTATGCCTCAC
GCATCCAGCGCCCCTTCTCCGTGAAG
TTCGACCCGTACACGCTGGCCATCGA
CGTGCTGGACAGCCCCCAGGCCGTGC
GGCGCTCCCTGGAGGGTGTCCAGGAT
GAGCTGGACACCCTTGCCCATGCGCT
GAGTGCCATTGGCGCAGAGAACCTCT
ACTTCCAATCGCACCATCATCACCAC
CATGATTACAAGGATGACGACGATAA
GTGAGGATCC

Final protein sequence (Tag sequence in lowercase):

MVPWFPRKVSELDKCHHLVTKFDPDL
DLDHPGFSDQVYRQRRKLIAEIAFQY
RHGDPIPRVEYTAEEIATWKEVYTTL
KGLYATHACGEHLEAFALLERFSGYR
EDNIPQLEDVSRFLKERTGFQLRPVA
GLLSARDFLASLAFRVFQCTQYIRHA
SSPMHSPEPDCCHELLGHVPMLADRT

FAQFSQDIGLASLGASDEEIEKLSTL
YWFTVEFGLCKQNGEVKAYGAGLLSS
YGELLHCLSEEPEIRAFDPEAAAVQP
YQDQTYQSVYFVSESFSDAKDKLRSY
ASRIQRPFSVKFDPYTLAIDVLDSPQ
AVRRSLEGVQDELDTLAHALSAIGae
nlyfq^shhhhhhdykddddk

^ TEV cleave site

Tags and additions: aenlyfq^shhhhhhdykddddk (^ - TEV cleavage site); C-terminal 6xHis-FLAG tag.

Host: BL21 (DE3) R3-pRARE2.

Growth medium, induction protocol: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50µg/ml kanamycin and 34µg/ml chloramphenicol. Several colonies were combined to inoculate a 1ml culture in TB (+ 50µg/ml kanamycin, 34µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v, and the resulting glycerol stock was frozen at -80°C in 100µl aliquots. A loopful of cells from the glycerol stock was inoculated into 20-ml of TB medium containing 50µg/ml kanamycin and 34µg/ml chloramphenicol and grown overnight at 37°C. 2x 1L TB medium (containing 50µg/ml kanamycin) were each inoculated with 10ml of the overnight culture and grown in 2.5L UltraYield baffled flasks until OD_{600} of 3.0. Cells were cooled to 18°C, IPTG added to 0.1mM and growth continued at 18°C overnight. The cells were collected by centrifugation then the pellets were scraped out and transferred to 50ml Falcon tubes and frozen at -80°C.
Lysis buffer: 50 mM Na-phosphate buffer, pH 7.5; 500 mM NaCl; 10 mM imidazole; 5% glycerol; 0.5 mM TCEP. 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase. 2x Lysis buffer contains the same components at double concentration.
Extraction buffer, extraction method: Frozen cell pellets (approx 35g) were thawed briefly in a bath of warm water (20 - 37°C) then transferred to ice. One volume (i.e. 1ml per gram of cells) of 2x lysis buffer was added, followed by 1x lysis buffer to a total volume of 50ml. The cells were resuspended by agitating and sonication (10 sec on, 10 sec off, 35% amplitude). Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, stirring for 15 minutes, then centrifugation for 20 minutes at 25,000 x g. The supernatant was then further clarified by filtration (Acrodisc filters, 0.2µm).

Column 1: Ni-Affinity, HisTrap Crude FF, 5ml (GE Healthcare).

Column 1 Buffer:
Affinity buffer: 50mM mM Na-phosphate buffer, pH 7.5; 500mM NaCl; 5% Glycerol; 10mM Imidazole; 0.5 mM TCEP.
Wash buffer: 50mM Na-phosphate buffer, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole; 0.5 mM TCEP.
Elution buffer: 50mM Na-phosphate buffer, pH 7.5; 500mM NaCl; 5% Glycerol; 300mM Imidazole; 0.5 mM TCEP.

Column 1 Procedure: The cell extract was loaded on the column at 4ml/minute on an ÄKTA-Express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4ml/minute. The eluted peak of A_{280} was automatically collected.

Column 2: Gel filtration, HiLoad 16/60 Superdex S200 prep grade, 120ml (GE Healthcare).

Column 2 Buffers: **Gel Filtration buffer:** 10 mM HEPES pH 7.5, 500 mM NaCl; 5% Glycerol; 0.5 mM TCEP.
Column 2 Procedure: The eluted fractions from the Ni-affinity HisTrap column was loaded on the gel filtration column in GF buffer at 1.2ml/minute. Eluted proteins were collected in 2ml fractions and analysed on SDS-PAGE and protein pooled.
Enzymatic treatment and purification: The C-terminal 6xHis-FLAG tag was cleaved by incubating the protein overnight at 4°C with TEV protease. Cleaved protein was purified by batch binding on 3ml pre-equilibrated 50% Ni-NTA bead solution (rotating at 4°C) for 1 hour then the column poured. The flow through fractions as well as fractions eluted with GF buffer containing 20 mM, 40 mM, 100 mM imidazole are collected, pooled and concentrated as below.
Protein concentration: The cleaved purified protein was concentrated in a VivaSpin4 centrifugal device (10 K MWCO) to 8.7 mg/ml and stored at 4°C. The protein concentration was determined spectrophotometrically using ε_{280} = 40340.
Mass spectrometry characterization: Observed mass of native protein was 39041 Da (calculated mass was 39039.1 Da without 6xHis-FLAG tag).
Crystallisation: Crystals were grown by vapour diffusion at 20°C, in 150nl sitting drops mixing 75nl protein and 75nl mother liquor (0.2 M ammonium acetate, 0.1M citrate pH 5.6, 30% PEG 4K) equilibrated against 20µl reservoir containing mother liquor. Crystals were cryo-protected in mother liquor containing 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.
Data Collection: **Resolution:** 2.68 Å **X-ray source:** Diamond Light Soucre beamline IO2.