Molecular Biology

Entry Clone Accession: IMAGE:3923041
Entry Clone Source: MGC
SGC Construct ID: GYG2A-c006
Protein Region: T4-A269 (S47D, V51R)
Vector: pNIC-CTHF
Tag: C-TEV;C-6HIS;C-FLAG
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MTDQAFVTLATNDIYCQGALVLGQSLRRHRLTRKLVVLITPQVSDLLRRILSKVFDEVIEV
NLIDSADYIHLAFLKRPELGLTLTKLHCWTLTHYSKCVFLDADTLVLSNVDELFDRGEFSA
APDPGWPDCFNSGVFVFQPSLHTHKLLLQHAMEHGSFDGADQGLLNSFFRNWSTTDIHK
HLPFIYNLSSNTMYTYSPAFKQFGSSAKVVHFLGSMKPWNYKYNPQSGSVLEQGSVSSS
QHQAAFLHLWWTVYQNNVLPLYKSVQAENLYFQSHHHHHHDYKDDDDK
Sequence after tag cleavage:
MTDQAFVTLATNDIYCQGALVLGQSLRRHRLTRKLVVLITPQVSDLLRRILSKVFDEVIEV
NLIDSADYIHLAFLKRPELGLTLTKLHCWTLTHYSKCVFLDADTLVLSNVDELFDRGEFSA
APDPGWPDCFNSGVFVFQPSLHTHKLLLQHAMEHGSFDGADQGLLNSFFRNWSTTDIHK
HLPFIYNLSSNTMYTYSPAFKQFGSSAKVVHFLGSMKPWNYKYNPQSGSVLEQGSVSSS
QHQAAFLHLWWTVYQNNVLPLYKSVQAENLYFQ
DNA Sequence:
ATGACTGATCAGGCTTTTGTCACACTAGCCACCAATGACATCTACTGCCAGGGCGCCC
TGGTCCTGGGGCAGTCACTGAGGAGACACAGGCTGACGAGGAAGCTGGTGGTGTTG
ATCACTCCTCAGGTGTCCGATCTGCTCAGGCGCATCCTCTCGAAGGTGTTCGATGAAG
TCATTGAAGTGAATCTAATCGATAGTGCCGACTACATCCACCTGGCCTTTCTGAAGAG
ACCTGAGCTCGGGCTCACCCTCACCAAGCTTCACTGTTGGACTCTCACTCACTACAGC
AAGTGTGTCTTCCTGGATGCAGACACTCTGGTGCTGTCCAATGTCGATGAGCTGTTTG
ACAGGGGAGAGTTTTCTGCGGCCCCGGACCCCGGATGGCCGGATTGCTTCAATAGCG
GGGTGTTTGTCTTCCAGCCTTCTCTCCACACGCATAAACTCCTGCTACAGCACGCCAT
GGAACACGGCAGCTTTGACGGGGCAGACCAAGGCTTACTGAATAGTTTCTTCAGGAA
CTGGTCGACCACAGACATCCACAAGCACCTGCCGTTCATCTATAACTTGAGTAGTAAC
ACGATGTACACTTACAGCCCTGCCTTCAAGCAATTCGGTTCCAGTGCAAAGGTCGTCC
ACTTTTTGGGGTCCATGAAACCTTGGAACTACAAGTACAATCCACAGAGTGGCTCGGT
GTTGGAGCAAGGCTCAGTGTCCAGCAGCCAGCACCAGGCGGCATTCCTTCATCTCTG
GTGGACGGTCTACCAGAACAACGTGCTGCCCCTTTATAAAAGCGTCCAAGCAGAGAA
CCTCTACTTCCAATCGCACCATCATCACCACCATGATTACAAGGATGACGACGATAAGT
GA

Protein Expression

Medium: Terrific Broth+ (50 g Merck TB, 20 g glycerol and water to 1 L) was autoclaved. After autoclaving the following were added to increase biomass, 1 mL of 1000x *E.coli* metals, 1 mL of 10 % Antifoam 204 (in ethanol), 1 mL of 1 M magnesium sulphate (autoclaved), 10 mL of 1 M ammonium sulphate (autoclaved) and 20 mL of 25 % glucose (autoclaved). 1000x *E.coli* Metals = 27 g/L $FeCl_2.6H_20$, 2 g/L $ZnCl_2.4H_20$, 2 g/L $CoCl_2.6H_20$, 2 g/L $Na_2MoO_4.2H_20$, 1 g/L $CaCl_2.2H_20$, 1 g $CuCl_2$, 0.5 g/L H_3BO_3, 100 mL 37 % HCl water to 1 L.
Antibiotics: Kanamycin (50 µg/mL) and Chloramphenicol (35 µg/mL)
Procedure: A 10 mL LB o/n culture was grown and used to inoculate 1 L of Terrific Broth+ in a 2.5 L Ultra Yield baffled flask. Cells were grown for 4 h at 37 °C 250 rpm shaking and then for 2

h at 18 °C. IPTG to a final concentration of 100 µM was added and cells grown o/n at 18 °C. Cells were harvested by centrifugation at 4,000 g for 20 minutes.

Protein Purification

Procedure: To 10 g of cell paste was added 20 mL of 40 mM tris.HCl, 10 % glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 7.5. After resuspending, lysozyme (0.5 mg/mL final concentration) and protease inhibitors (Roche cOmplete mini EDTA free) were added and cells incubated for 30 minutes at RT before freezing at -80 °C. After thawing, Triton X-100 and NP-40 were added to 1 % and 0.2 % final concentration respectively. After a 30 minute incubation at RT $MgCl_2$ and $CaCl_2$ were added to 1 mM and 0.13 mM respectively followed by bovine DNase to a final concentration of 25 µg/mL. After 30 minutes a further addition of bovine DNase was added and lysed cells incubated for a further 30 minutes. Lysate was then centrifuged at 20,000 g for 30 minutes at 4 °C and the supernatant added to 5 mL of profinity resin (BioRad) in a 50 mL Falcon tube, resin having been pre-equilibrated in WASH buffer (20 mM HEPES, 500 mM NaCl, 5 % glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 7.5). Resin was mixed end over end with the soluble fraction for 30 minutes at 4 °C and then tipped in to a Zeba spin column (Pierce). Resin was washed with 50 mL of WASH buffer and then 30 mL of the same but with 30 mM imidazole. Resin was then washed with 15 mL of WASH buffer with 30 mM imidazole and 0.1 M arginine. Protein was eluted with WASH buffer plus 500 mM imidazole and 0.1 M arginine and 1 mL fractions collected. To keep protein soluble, to the peak fractions pool was added an equal volume of STORAGE buffer (10 mM MES, 500 mM NaCl, 20 % glycerol, pH 6 + 0.4 M arginine) and protein concentrated to 15 mg/mL. 1 part TEV was added to 10 parts GYG2A and the sample incubated o/n at 4 °C. Cleaved protein was diluted 5 fold in WASH buffer and run back over the Profinity resin to remove TEV-his and any uncleaved protein. Protein was exchanged into STORAGE buffer with 0.2 M arginine and concentrated to 13.6 mg/mL

Columns: Column 1: IMAC; Column 2: -ve IMAC;
Concentration: 13.6 mg/ml
Mass-spec Verification: Yes
Compound Exact Mass: expected 31153.6 Da, observed 31023.89 Da (-129.71 indicating N-term Met Loss)

Structure Determination

Crystallization:
Crystallization Condition: 0.3M magnesium formate, 0.1M tris pH 8.5*;*
Protein Concentration: 13.6mg/ml*;*
Data Collection: *Beamline:* Dmnd I03; *Resolution:* 1.93 Å
Data Processing: Xia2 autoprocessing; Phenix refine