Materials and Method

Note: To our best knowledge, this should represent an accurate description of the materials and methods required to reproduce our work. If any of the content on this page is difficult to interpret or should you have trouble repeating our work, do not hesitate to contact us as soon as possible in order for us to provide additional information and advice.

Entry Clone Source: MGC
Entry Clone Accession: IMAGE:6064429
SGC Construct ID: PTDSRB-c200
GenBank GI number: gi|45219814
Vector: pET-28a(+)
Final protein sequence: mgsshhhhhhssglvprgshMNHKSKKRI REAKRSARPELKDSLDWTRHNYYESFSLS PAAVADNVERADALQLSVEEFVERYERPY KPVVLLNAQEGWSAQEKWTLERLKRKYRN QKFKCGEDNDGYSVKMKMKYYIEYMESTR DDSPLYIFDSSYGEHPKRRKLLEDYKVPK FFTDDLFQYAGEKRRPPYRWFVMGPPRSG TGIHIDPLGTSAWNALVQGHKRWCLFPTS TPRELIKVTRDEGGNQQDEAITWFNVIYP RTQLPTWPPEFKPLEILQKPGETVFVPGG WWHVVLNLDTTIAITQNFASSTNFPVVWH KTVRGRPKLSRKWYRILKQEHPELAVLAD SVDLQESTGIASDSS
Tag sequence: N-terminal His6 tag, followed by a thrombin protease cleavage site: mgsshhhhhhssglvpr*gsh (* - thrombin cleavage site)
Tag removed: no
Host: BL21(DE3)-R3-pRARE2 (previously known as Rosetta)
Expression protocol: A glycerol stock of *E.coli* host strain Rosetta carrying the expression plasmid was used to inoculate 10 ml of media supplemented with 25 µg/ml kanamycin and 30 µg/ml chloramphenicol. This starter culture was grown overnight at 37°C and used to inoculate 4L Le Master media prepared with 50 mg/l L-selenomethionine instead of L-met and supplemented with 25 µg/ml kanamycin and 30 µg/ml chloramphenicol. Cells were grown at 37 °C until they reached an OD_{600} of 0.6; the temperature was then reduced to 25 °C and IPTG was added at a final concentration of 0.5 mM. Cells were harvested 16-18 hours post-induction and stored at -80°C.
Lysis buffer: 50 mM Tris, pH 7.5, 200 mM NaCl, 10 mM imidazole, 10% glycerol**,** 0.2 mM TCEP.
Procedure: Cell pellets were resuspended in 150 ml lysis buffer supplemented with DnasI and lysed by sonication using a Sonics Vibracell sonicator and five 30 s bursts at 60%. Cell debris was removed by centrifugation at 20,000 rpm for 30 min. at 4°C.
Column 1: HisTrap HP 5ml (GE Healthcare)
Buffers: **Lysis buffer:** 50 mM Tris, pH 7.5, 200 mM NaCl, 10 mM imidazole, 10% glycerol**,** 0.2 mM TCEP **Ni-NTA Elution buffer:** 50 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol**,** 0.2 mM TCEP, 500 mM imidazole **Storage Buffer:** 50 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol**,** 0.2 mM TCEP.

Procedure: The clarified supernatant was loaded onto a HisTrap column at 2 ml/min using an Akta purifier. The column was developed using a gradient from 0 to 100% elution buffer. Fractions were analysed by SDS-PAGE and those containing protein of the correct molecular weight and the best purity were pooled and concentrated using a 10,000 MWCO filter (Millipore).
Protein concentration: The protein solution was buffer-exchanged into storage buffer using a 10,000 MWCO filter and concentrated to 19 mg/ml measured by a NanoDrop ND-1000 spectrophotometer. Protein was aliquoted and stored at -80°C.
Crystallization: Crystals were grown at 4°C by vapour diffusion in sitting drops by mixing protein (19 mg/ml) and well solution containing 0.8M NaH_2PO_4, 0.8M KH_2PO_4, 0.1M NaHEPES pH 7.5 at a protein to precipitant ratio of 1:2. A crystal was cryo-protected using well solution supplemented with 30% (v/v) glycerol and flash-cooled in liquid nitrogen.
Data Collection: Resolution (scaled): 1.75 Å; **X-ray source:** Synchrotron - Diamond I04.