Molecular Biology

Entry Clone Accession: IMAGE:3048375
Entry Clone Source: MGC
SGC Construct ID: DCLRE1AA-c081
Protein Region: K698-Y1040
Vector: pFB-LIC-Bse. This is a baculovirus transfer vector (Bac-to-bac), with N-terminal 6 His tag followed by a TEV cleavage site
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMKKTCPFYKKIPGTGFTVDAFQYGVVEGCTAYFLT
HFHSDHYAGLSKHFTFPVYCSEITGNLLKNKLHVQEQYIHPLPLDTECIVNGVKVVLLDA
NHCPGAVMILFYLPNGTVILHTGDFRADPSMERSLLADQKVHMLYLDTTYCSPEYTFPSQ
QEVIRFAINTAFEAVTLNPHALVVCGTYSIGKEKVFLAIADVLGSKVGMSQEKYKTLQCL
NIPEINSLITTDMCSSLVHLLPMMQINFKGLQSHLKKCGGKYNQILAFRPTGWTHSNKFT
RIADVIPQTKGNISIYGIPYSEHSSYLEMKRFVQWLKPQKIIPTVNVGTWKSRSTMEKYFR
EWKLEAGY
Sequence after tag cleavage:
SMKKTCPFYKKIPGTGFTVDAFQYGVVEGCTAYFLTHFHSDHYAGLSKHFTFPVYCSEIT
GNLLKNKLHVQEQYIHPLPLDTECIVNGVKVVLLDANHCPGAVMILFYLPNGTVILHTG
DFRADPSMERSLLADQKVHMLYLDTTYCSPEYTFPSQQEVIRFAINTAFEAVTLNPHALV
VCGTYSIGKEKVFLAIADVLGSKVGMSQEKYKTLQCLNIPEINSLITTDMCSSLVHLLPM
MQINFKGLQSHLKKCGGKYNQILAFRPTGWTHSNKFTRIADVIPQTKGNISIYGIPYSEHS
SYLEMKRFVQWLKPQKIIPTVNVGTWKSRSTMEKYFREWKLEAGY
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGAAAAAGACATGTCCATTCTATAAGAAAATACCTGGAACCGGCTT
TACAGTTGATGCCTTTCAGTATGGCGTGGTTGAAGGTTGCACAGCCTATTTTCTCACAC
ATTTTCATTCTGATCATTATGCTGGATTGTCTAAACACTTCACATTTCCAGTTTATTGTA
GTGAGATAACTGGCAATTTGTTGAAGAACAAGCTTCATGTGCAAGAACAATATATTCA
CCCATTGCCACTGGACACTGAATGTATTGTGAATGGTGTCAAAGTTGTTTTGCTTGATG
CCAATCACTGTCCAGGTGCTGTCATGATCCTCTTTTATCTTCCTAATGGTACTGTCATAT
TACACACGGGAGACTTCAGAGCAGATCCCAGCATGGAACGTTCTCTTCTTGCGGACC
AGAAAGTCCATATGCTGTACTTAGATACCACATATTGTAGCCCAGAATACACCTTTCCA
TCTCAGCAAGAGGTTATCCGGTTTGCCATCAACACTGCCTTTGAGGCTGTAACTCTAA
ACCCACATGCTCTTGTTGTCTGTGGCACTTACTCTATTGGAAAAGAGAAAGTCTTCCT
AGCCATTGCTGATGTTTTAGGTTCAAAAGTGGGCATGTCCCAGGAAAAATATAAAACT
CTACAGTGCCTCAATATACCAGAAATTAATTCACTCATCACTACCGACATGTGCAGTTC
ATTGGTTCACCTTCTCCCAATGATGCAAATTAATTTTAAGGGCTTACAGAGTCATTTGA
AGAAGTGTGGTGGGAAATACAATCAGATTTTGGCATTTCGACCTACAGGATGGACACA
CTCTAACAAGTTCACTAGAATAGCAGATGTTATTCCCCAGACCAAAGGAAACATTTCA
ATATATGGAATTCCTTACAGTGAACACAGCAGCTACCTAGAAATGAAGCGCTTTGTCC
AGTGGCTGAAGCCCCAGAAAATCATACCTACTGTAAATGTGGGCACCTGGAAATCTAG
GAGCACAATGGAGAAATATTTTAGAGAGTGGAAATTGGAAGCTGGATATTGACAGTAA
AGGTGGATACGGATCCGAATTCGAGCTCCGTCGACAAGCTT

Protein Expression

Medium: SF900II
Antibiotics: Ampicillin
Procedure: Baculoviruses were generated by recombination in *E. coli* DH10Bac (Life

Technologies) followed by transfection and two rounds of amplification in SF9 cells. DCLRE1A was expressed in 1-L cultures of SF9 cells in 4-L shaker flasks at 27°C, infected at 2 × 10^6 cells/ml with 3 ml of virus, and incubated for further 70 h. The cells were collected by centrifugation, suspended in 15 ml/l of lysis buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 5% v/v glycerol, 10 mM imidazole, and 1 mM TCEP) and frozen at −80°C.

Protein Purification

Procedure: Cells were thawed, 3–5 volumes of lysis buffer were added, and the cells were disrupted by sonication. The lysate was centrifuged for 30 min at 40 000 × g, and the clear supernatant was collected. The clarified cell lysate was loaded on a 5-ml NiNTA column by gravity flow. The column was washed with 20 volumes of wash buffer (lysis buffer with 30 mM imidazole), and the protein was recovered with elution buffer (lysis buffer with 300 mM imidazole). The eluted protein was combined with His10-tagged TEV protease (1/10 w/w) in a dialysis tubing, and digestion of the tag was performed overnight at 6°C while dialysing against 4 l of wash buffer. The material was then passed through a 1-ml HisTrap column to remove contaminating proteins and remaining TEV protease. The column was developed with a 20-ml gradient from wash buffer to elution buffer, and all fractions were analyzed by SDS-PAGE.

The DCLRE1A containing fractions from the second IMAC column were combined, concentrated to <4 ml using a centrifugal concentrator (MWCO = 10 kDa), and loaded on a Superdex S200 HR 16/60 column equilibrated with GF buffer (10 mM HEPES, pH 7.5, 300 mM NaCl, 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP), 5% glycerol) at 1.2 ml/min.

The protein was confirmed by ESI-TOF intact mass spectrometry (Predicted: 39092.5 observed: 39093.8)

Structure Determination

Crystallization: Protein crystallization was performed by vapour diffusion in sitting drops at 4°. A protein solution at 9-10 mg/mL was mixed at with an equal volume crystallization solution containing 30% PEG 1000, 0.1M MIB pH 6.0 (MIB is Sodium malonate dibasic monohydrate, Imidazole, Boric acid). The crystals were backsoaked overnight in a solution containing 30% PEG 1000, 0.1 M Hepes pH 7.0 to remove the malonate ion from the active site and were subsequently soaked overnight in a solution containing an additional 20 mM ceftriaxone before being loop mounted and plunged directly into a pool of liquid nitrogen.

Data Collection: Data was collected to 2.4Å resolution at ESRF beamline BM30B, and processed using XDS.
Data Processing: The structure was solved by molecular replacement using the program MOLREP and PDBid 5AHO as a search model. Refinement was performed using REFMAC to a final Rfactor = 22.6%, Rfree = 31.5%.