HECTD1

PDB:2LC3
Entry Clone Accession:hectd1.BAA86445.KZA.KIAA1131
Entry Clone Source:pBlueScript SK+
SGC Clone Accession:hectd1.1881-1968.202B08 (SDC202B08)
Tag:MHHHHHHSSGRENLYFQG
Host: Competent BL21 (DE3) cells (Invitrogen, C6000-03)
Vector:pET28MHL (GenBank, EF456735)

Sequence:

MHHHHHHSSGRENLYFQGMKDSDKEKENGKMGCWSIEHVEQYLGTDELPKNDLITYLQ
KNADAAFLRHWKLTGTNKSIRKNRNCSQLIAAYKDFCEHGTKSGLNQG

Growth

Medium: M9 minimal media (100 uM $ZnSO_4$, 8.55 mM NaCl, 47.6 mM Na_2HPO_4, 22 mM KH_2PO_4, 100 mM $MgSO_4$, 2 mM biotin, 1.5 mM thiamine.HCl, 10 mM $ZnSO_4$, and 0.1 M $CaCl_2$) supplemented with $^{15}NH_4Cl$, ^{13}C6-D-glucose, and 50 µg/ml kanamycin
Procedure: Competent BL21 (DE3) cells (Invitrogen, C6000-03) were transformed and incubated overnight (18 hours) at 220 rpm at 37°C in a 125 ml flask containing 50 ml of M9 minimal media (100 uM $ZnSO_4$, 8.55 mM NaCl, 47.6 mM Na_2HPO_4, 22 mM KH_2PO_4, 100 mM $MgSO_4$, 2 mM biotin, 1.5 mM thiamine. HCl, 10 mM $ZnSO_4$, and 0.1 M $CaCl_2$) supplemented with $^{15}NH_4Cl$, ^{13}C6-D-glucose, and 50 µg/ml kanamycin. The overnight starter culture was transferred to a 2 L flask containing 1 L of M9 minimal media supplemented with supplemented with $^{15}NH_4Cl$, ^{13}C6-D-glucose, and 50 µg/ml kanamycin, and incubated at 37°C. When the OD(600) reached a value of 1.0, protein expression was induced with 100 µM isopropyl-thio-ß-D-galactopyranoside and the cells were incubated overnight (15.5 hours) at 220 rpm at 15°C. Cell pellets were collected by centrifugation (7000 rpm, 20 mins) and frozen in 50 mL Falcon tubes at -80°C for storage.

Purification

Procedure:
The lysate was clarified by centrifugation for 20 min at 4°C. The supernatant was mixed with 2 mL of Ni2+ affinity beads per 40 mL lysate. The mixture was incubated with mixing for 20 minutes at 4°C. The lysate was spun at 2000 rpm for 6 minutes, and the supernatant was decanted. The remaining resin was resuspended and washed twice with lysis buffer, followed by two washes with with 5 mL of cold wash buffer. The washed resin was transferred to a gravity filter column and further washed with 2 mL of wash buffer. Samples were eluted from the resin by exposure to 5 mL of elution buffer. The purified protein was dialyzed with TEV protease against cutting buffer overnight at room temperature. Cleaved his-tag was removed by binding with 2 mL Ni^{2+} affinity beads.

The purified protein was exchange from elution buffer into Tris-based NMR buffer by ultracentrifugation using 5 mL concentrators with a 5,000 molecular weight cut-off (VivaSpin 2

MES) at 3000 rpm, resulting in a final volume of 300 µl (final protein concentration of 0.5 mM). The concentrated protein was transferred to a 5 mm Shigemi NMR tube.

Extraction

Procedure:
The frozen cell pellet stored in a 50 ml Falcon tube obtained from 1L of culture was thawed by soaking in warm water, and resuspended in 40 mL lysis buffer. The cell pellet was lysed by sonication (Branson Sonicator) on ice for 10 minutes total sonication time (10 sec pulses at half-maximal frequency with 10 second rest).

MassSpec:A series of spectra (3D 1H-13C NOESY, 3D 1H-15N NOESY, 2D 1H-13C Constant Time HSQC, 3D HNCO, 3D HNCA, 3D CBCA(CO)NH, 3D HBHA(CO)NH, 3D (H)CCH-TOCSY, and 3D H(C)CH-TOCSY) were generated using a 500MHz Bruker AVANCE spectrometer and a 800MHz Bruker AVANCE spectrometer. NMR data was processed and analyzed using NMRPipe, MDDGUI, Sparky, FMCGUI, TALOS, CYANA, CNS, and PSVS.