

Cp-MDH

PDB:2HJR

Revision

Revision Type:created

Revised by:created

Revision Date:created

Entry Clone Accession:cgd7-470

Entry Clone Source:Cryptosporidium parvum strain Iowa genomic DNA

SGC Clone Accession:

Tag:

Host:BL21(DE3)-R3

Construct

Prelude:

Sequence:

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mgsshhhhhhssgrenlyfqgLKKISYKYNTVIMRKKISIIGAGQIGSTIALLGQKDLGDVYMFIDIEGVPQGKALDLNHCMALI
GSPAKIFGENNYEYLQNSDVIITAGVPRKPNMTRSDLLTVNAKIVGSVAENVGKYCPNAFVICITNPLDAMVYYFKEKSGIPANKV
CGMSGVLDsarFRCNLSRALGVKPSDVSAIVVGGHGDEMIPLTSSVTIGGIILSDFVEQGKITHSQINEIIKKTAFGGGEIVELLKT
GSAFYAPAAASAVAMAQAYLKDSKSVLVCSTYLTGQYNVNNLFVGVPVIGKNGIEDVVIVNLSDEKSLFSKSVESIQNLVQDLKSL
NL
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Vector:p15-tev-LIC

Growth

Medium:

Antibiotics:

Procedure:Cp-MDH was expressed in E. coli BL21-(DE3)-R3 cells in Terrific Broth (TB) in the presence of kanamycin/chloramphenicol (50 µg/mL and 25 µg/mL respectively). A single colony was inoculated into 10 mL of LB with of kanamycin/chloramphenicol (50 µg/mL and 25 µg/mL respectively) in a 125 mL flask and incubated with shaking at 250 rpm overnight at 37 °C. The culture was transferred into 100 mL of TB with 50 µg/mL kanamycin in a 250 mL shaking flask and incubated at 37 °C for 3 hours. The culture was transferred into 2 X 1.8 L TB with 50 µg/mL kanamycin and 0.3 mL of antifoam (Sigma) in 2 L bottles and cultured using the LEX system to an OD600 of 2.5. The culture was cooled to 15 °C, and isopropyl-1-thio-D-galactopyranoside (IPTG) was added to 0.4 mM, and the culture was incubated overnight at 15 °C.

Purification

Procedure

The cleared lysate was loaded onto a column prepacked with 10 g DE52 (Whatman) anion exchange resin (previously activated with 2.5 M NaCl and equilibrated with Binding Buffer); and

subsequently onto a 1.0 - 2.5 mL Ni-NTA (Qiagen) column pre-equilibrated with Binding Buffer at approximately 1 - 1.5 mL/min. The volume of the Ni-NTA resin was pre-determined by the predicted protein yield from test expression analysis. After the lysate was loaded, the DE52 was further washed with 20 mL of Binding Buffer (added 100ul Protease inhibitor). Each Ni-NTA column was then washed with 200 mL of Wash Buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM imidazole, and 5 % glycerol) at 20 mL/min. After washing, the protein was eluted with 15 mL of Elution Buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 250 mM imidazole, and 5 % glycerol). EDTA was immediately added to the elution fraction to 1 mM; and DTT was added to 1-5 mM after approximately 15 more minutes.

The Ni-NTA purified protein was loaded onto a 26/60 S200 Superdex gel filtration column; and the major peak corresponding to the CP-MDH was collected and concentrated using a 15 mL Amicon Ultra centrifugal filter device (Millipore). The concentrated protein was stored at 4 °.

Tag cleavage: Add 5ul of in house purified TEV to 20mg of your protein, then add 1mM DTT. The concentration of protein should be 4mg/ml (you can dilute your protein by binding buffer), then keep at 4 degC overnight. set up Bio-Rad Econo (1.5 x 15 cm x 27 mL) columns with 1.5 mL of suspended Ni-NTA Superflow resin. Equilibrate the columns with 50 mL of Binding Buffer and let drain completely by gravity. Determine the percentage of His-tag cleavage from the molecular weights of the cut and uncut protein samples on the SDS gel or you can run mass spect. Apply the protein samples onto the equilibrated Ni-NTA Superflow resin (2ml) and collect the flow-through. Then add more Binding Buffer to each column and check by assaying 10 microL of each sample in 100 microL of Bradford reagent. Add 10 mL of Wash Buffer if protein does not come off the column in Binding Buffer.

Extraction

Procedure

The culture was harvested by centrifugation and the cell pellet was suspended in 160 mL of binding buffer (50 mM HEPES, pH 7.5, 0.5 M NaCl, 5% glycerol, and 15 mM imidazole) with protease inhibitor (1 mM benzamidine-HCl and 1 mM phenylmethyl sulfonyl fluoride, PMSF) and kept in 50 mL Falcon tubes at 80 °C. Before purification, the cell suspension was thawed overnight at 4 °C. Prior to mechanical lysis, each tube of cell suspension was pretreated with 0.5 % CHAPS and 500 units of benzonase (per 40 mL of resuspended cell pellet) for 40 minutes at room temperature. Then the cells were mechanically lysed with a microfluidizer (Microfluidizer Processor, M-110EH) at approximately 18000 psi. The lysate was centrifuged at 24000 rpm for 20 minutes at 10 °C.

Concentration:

Ligand

MassSpec:

Crystallization: Purified Cp-MDH was crystallized using the hanging drop vapor diffusion method in a 24-well plate. A solution containing 15% PEG4K, 15% PEG400, 0.1M Sodium citrate pH 5.6, 0.2M Ammonium acetate were used as the mother liquor. Mother liquor (500 µL) was added to the buffer reservoir, and 1 µL protein solution that incubated with 10mM NAD for 1 hour on ice was mixed with 1µL mother liquor. The plate was kept at 18 °C. The crystals appeared overnight but took two days to grow to full size.

NB. It was found the NAD had hydrolyzed to ADP-ribose in the crystallization buffer.

NMR Spectroscopy:

Data Collection:

Data Processing: