AASDHGPPT

Entry Clone Source: MGC

Entry Clone Accession: IMAGE:4081713

SGC Construct ID: AASDHPPTA-c003

GenBank GI number: gi|20357568

Vector: pCOEX-1

Tags and additions (His 6 + TEV cleavage site):
mgsshhhhhhssgrenlyfqgh

Host : *E.coli* BL21 DE3-R3

Construct coding sequence:
mgsshhhhhhssgrenlyfqghMEGVRW
AFSCGTWLPSRAEWLLAVRSIQPEEKERI
GQFVFARDAKAAMAGRLMIRKLVAEKLNI
PWNHIRLQRTAKGKPVLAKDSSNPYPNFN
FNISHQGDYAVLAAEPELQVGIDIMKTSF
PGRGSIPEFFHIMKRKFTNKEWETIRSFK
DEWTQLDMFYRNWALKESFIKAIGVGLGF
ELQRLEFDLSPLNLDIGQVYKETRLFLDG
EEEKEWAFEESKIDEHHFVAVALRKPDGS
RHQDVPSQDDSKPTQRQFTILNFNDLMSS
AVPMTPEDPSFWDCFCFTEEIPIRNGTKS

Growth medium, induction protocol: Medium: TB + 34 µg/ml Chloramp. Cells were grown in 1 liter TB in 2.5-L baffled flasks, which was inoculated with 10 ml overnight culture. The culture was grown at 37°C to OD=2.3, and transfered to 25°C. 1 mM IPTG was then added, and incubation continued for 4 hours. The cells were then collected by centrifugation and frozen at -80°C.

Extraction buffer, extraction method: Lysis buffer: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP, Complete® protease inhibitors (1 tablet/50 ml).

Frozen cell pellets were thawed on ice over night and resuspended in a total volume of 40 ml lysis buffer, the cells were disrupted by high pressure (20 psi) followed by sonication.

Nucleic acids and cell debris were removed by adding 0.15% PEI from a 5% (w/v) stock, stirring for 15 minutes, then centrifugation for 20 minutes at 40,000xg. The supernatant was then further clarified by filtration (0.45 µm).

Column 1 : Ni-affinity, HisTrap, 1 ml (GE/Amersham)

Buffers: Lysis buffer: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole, 0.5 mM TCEP. **Wash buffer:** 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM imidazole, 0.5 mM TCEP. **Elution buffer:** 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole, 0.5 mM TCEP.

Procedure: The cell extract was loaded on the column at 0.8 ml/minute on an AKTA-express system (GE/Amersham). The column was then washed with 10 volumes of Lysis buffer, 10

volumes of wash buffer, and then eluted with elution buffer at 0.8 ml/min. The eluted peak of A_{280} was automatically collected.

Column 2 : Gel filtration, Hiload 16/60 Superdex 200 prep grade 120 ml (GE Healthcare)

Buffers : GF buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5 % glycerol, 0.5 mM TCEP.

Procedure: The eluted fractions from the Ni-affinity Histrap columns were loaded on the gel filtration column in GF buffer at 1.0 ml/min. Eluted proteins were collected in 1 ml fractions.

Concentration : In vivaspin 6 ml 10 K. Concentration determined from the absorbance at 280 nm using the extinction coefficient and the nanodrop method.

Concentration : 19.6 mg/ml

Enzymatic treatment : no cleavage

Mass spectrometry: observed mass corresponds to predicted protein sequence

ACP

Target ID: ACP domain of FASNA

Entry clone source: An ACP fragment was amplified from a human liver cDNA libaray and cloned into a bacterial expression vector. Site directed mutagenesis of the phosphopantetheinyl acceptor site (Ser39) was exchanged to Ala using PCR .

Vector: pQE 80 L (Qiagen).

Sequence and Tags/ additions:
(His-tag and TEV protease site in lowercase)
mrgshhhhhhgsdydipttenlyfqgtRD
RDSQRDLVEAVAHILGIRDLAAVNLDSSL
ADLGLDALMSVEVRQTLERELNLVLSVRE
VRQLTLRKLQELSSKADEASELACPTPKE

Host : *E. coli* BL21-codon plus.

Growth medium, induction protocol: Cells were grown at 37°C in LB medium +100µg/ml carbenicillin to *A* 600 of 0.5-0.7 and induced with 1 mM IPTG for 3 h.

Extraction buffer, extraction method: Cells were suspended in 0.25 M potassium phosphate buffer, pH 7, containing 10% glycerol 5 mM mercaptoethanol and protease inhibitors (leupeptin 5µg/ml, trans-epoxysuccinyl LGB 10µM, pepstatin 1µg/ml, and antitrypsin 5µg/ml final concentration), and lysed by four disruption cycles in a microfluidizer. The lysate was centrifuged at 50,000 *g* for 1 h at 4 °C, the supernatant was collected and filtered (0.4µ).

Column 1 : Ni-NTA affinity

Procedure: The soluble cell extract was loaded onto a NiNTA column at 0.5 ml/min and the column eluted successively with 10 vol buffer A (0.1 M potassium phosphate buffer, pH 7/2 mM mercaptoethanol, 10% glycerol) and 10 vol buffer B (Buffer A containing 125 mM imidazole. fractions were analyzed by SDS - PAGE and those containing ACP were pooled, DTT was added to 2 mM, final concentration and the sample was dialyzed overnight against 50 mM Tris-HCl pH 7.5/10% glycerol/1 mM DTT.

Column 2 : Resource-Q anion exchange

Procedure: The dialysate was filtered (0.4 μ) and applied at 2-ml per min to a column (5 ml) of Resource-Q equilibrated with 50 mM Tris-HCl pH 7.5/10% glycerol/1 mM DTT and eluted with a gradient containing 0-0.25 M NaCl. The major A280-absorbing zone was collected and a portion was subjected to cleavage with TEV protease.

Concentration : 10 mg/ml

Enzymatic treatment : ACP was treated with AcTEV protease (Invitrogen), 10 U/mg ACP for 24 h at 20°C followed by 40 h at 4°C, then reapplied to a Ni-NTA affinity column. This time the ACP , lacking the His-tag, eluted in the wash buffer while the AcTEV protease and the cleaved N-terminal peptide remained bound to the column. The sample was then dialyzed against 50 mM Tris pH 7.4/0.3M NaCl/1 mM DTT/10% glycerol and concentrated using a Vivaspin device (5k Da cutoff).

Mass spec characterization : Both the purified TEV-cleaved and not-cleaved ACPs were homogeneous, as evaluated by SDS - PAGE and Coomassie staining. The molecular masses of the TEV-cleaved and not-cleaved ACPs, determined by MALDI-MS, were 10,053.4 and 13,053.3 Da, respectively, close to the expected values. Protein concentration was estimated using the BioRad BCA method with bovine serum albumin as standard.

Common steps

Crystallisation: ACP , CoA, $MgCl_2$ and AASDHPPT were combined in the following ratios: 0.365 mM AASDHPPTA, 0.398 mM fACP (5% excess), 2.43 mM CoA and 12.1 mM $MgCl_2$. Crystals were obtained at 0.35 M $(NH_4)H_2PO_4$. A single crystal was transferred to a cryo-protectant prepared with 25% glycerol, 75% well solution and flash-cooled in liquid nitrogen.

Data acquisition and analysis: A suitable crystal was mounted with a loop and diffraction images were collected on a Rigaku FRE rotating anode, Osmic HR multilayer optics and a Rigaku HTC image plate detector. Indexing, integration and scaling were performed using the package XDS. The structure was solved with molecular replacement employing the AASDHPPT structure (PDB ID 2BYD) as search model using the program Phaser. No sufficiently similar model was found for the ACP , which was built de novo into the electron density map. Refinement was carried out with refmac5 and converged to a final R/Rfree of 0.192/0.247.