Molecular Biology

Entry Clone Accession: clone 27 (T615S mutation)
Entry Clone Source: Collaborator
SGC Construct ID: AASSA-c013
Protein Region: L455-P926
Vector: pFB-LIC-Bse
Tag: N-6HIS;N-TEV
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMALPDKYKYIQTLRESRERAQSLSMGTRRKVLVLG
SGYISEPVLEYLSRDGNIEITVGSDMKNQIEQLGKKYNINPVSMDICKQEEKLGFLVAKQD
LVISLLPYVLHPLVAKACITNKVNMVTASYITPALKELEKSVEDAGITIIGELGLDPGLDHM
LAMESIDKAKEVGATIESYISYCGGLPAPEHSNNPLRYKFSWSPVGVLMNVMQSATYLLD
GKVVNVAGGISFLDAVTSMDFFPGLNLEGYPNRDSTKYAEIYGISSAHTLLRGTLRYKGY
MKALNGFVKLGLINREALPAFRPEANPLTWKQLLCDLVGISPSSEHDVLKEAVLKKLGGD
NTQLEAAEWLGLLGDEQVPQAESILDALSKHLVMKLSYGPEEKDMIVMRDSFGIRHPSG
HLEHKTIDLVAYGDINGFSAMAKTVGLPTAMAAKMLLDGEIGAKGLMGPFSKEIYGPILE
RIKAEGIIYTTQSTIKP
Sequence after tag cleavage:
SMALPDKYKYIQTLRESRERAQSLSMGTRRKVLVLGSGYISEPVLEYLSRDGNIEITVGSD
MKNQIEQLGKKYNINPVSMDICKQEEKLGFLVAKQDLVISLLPYVLHPLVAKACITNKVN
MVTASYITPALKELEKSVEDAGITIIGELGLDPGLDHMLAMESIDKAKEVGATIESYISYCG
GLPAPEHSNNPLRYKFSWSPVGVLMNVMQSATYLLDGKVVNVAGGISFLDAVTSMDFFP
GLNLEGYPNRDSTKYAEIYGISSAHTLLRGTLRYKGYMKALNGFVKLGLINREALPAFRP
EANPLTWKQLLCDLVGISPSSEHDVLKEAVLKKLGGDNTQLEAAEWLGLLGDEQVPQAE
SILDALSKHLVMKLSYGPEEKDMIVMRDSFGIRHPSGHLEHKTIDLVAYGDINGFSAMAK
TVGLPTAMAAKMLLDGEIGAKGLMGPFSKEIYGPILERIKAEGIIYTTQSTIKP
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGGCATTACCTGATAAATATAAATATATCCAGACACTCCGGGAGAG
CAGGGAACGTGCTCAGTCACTTTCAATGGGCACCAGGAGAAAGGTTTTGGTTCTTGG
ATCTGGCTACATATCTGAGCCTGTATTAGAATATTTATCAAGAGATGGCAATATAGAAAT
AACAGTAGGATCTGACATGAAGAATCAAATTGAACAGTTAGGCAAGAAATATAATATT
AATCCTGTTAGCATGGACATTTGTAAACAAGAAGAGAAGCTGGGCTTCTTGGTGGCA
AAACAGGATCTTGTCATCAGCTTGTTGCCTTATGTATTGCACCCTCTTGTGGCCAAGGC
CTGCATCACAAACAAAGTTAACATGGTCACTGCAAGCTACATCACACCAGCACTAAA
AGAATTGGAAAAGAGTGTGGAAGATGCTGGCATCACAATCATTGGTGAATTGGGATTG
GACCCTGGTCTGGATCACATGTTAGCAATGGAATCAATAGATAAAGCCAAGGAAGTGG
GAGCCACGATTGAATCATATATTTCCTACTGTGGTGGGCTTCCAGCCCCTGAACATTCA
AACAATCCATTGAGATATAAATTTAGCTGGAGTCCAGTGGGAGTTTTGATGAATGTAAT
GCAGTCTGCCACCTATCTGCTCGATGGAAAGGTTGTGAATGTTGCAGGAGGCATCTCC
TTTCTTGATGCCGTTACGTCCATGGATTTTTTTCCAGGATTAAATTTGGAAGGCTATCCT
AACAGAGACAGTACGAAATATGCTGAGATTTATGGCATTTCTTCTGCTCACACTTTGTT
GCGGGGGACACTGAGATATAAGGGATATATGAAAGCTTTGAATGGATTTGTAAAATTA
GGTCTTATAAACAGAGAAGCGCTTCCTGCCTTTAGACCTGAGGCCAACCCTCTCACCT
GGAAACAACTCCTCTGTGACCTAGTTGGGATTTCACCCTCCTCTGAGCATGATGTGTT
GAAGGAAGCTGTTCTTAAGAAACTAGGAGGAGACAATACCCAGTTGGAGGCTGCTGA
ATGGTTGGGCTTACTTGGGGATGAACAAGTTCCTCAGGCAGAGTCCATTCTGGATGCC
CTCTCCAAGCATTTGGTCATGAAGCTTTCCTATGGTCCTGAAGAAAAAGATATGATTGT
GATGAGAGACAGCTTTGGAATCAGACATCCTTCTGGACATTTAGAACATAAAACGATT

GATCTTGTGGCTTATGGGGACATCAATGGCTTTTCAGCCATGGCTAAAACCGTGGGGT
TACCCACCGCCATGGCAGCCAAAATGTTGCTTGATGGTGAAATTGGAGCCAAAGGCC
TAATGGGGCCCTTTTCAAAGGAGATCTATGGACCAATATTGGAGCGAATTAAAGCAGA
AGGCATTATATATACTACACAGAGTACAATTAAACCATGACAGTAAAGGTGGATACGGA
TCCGAATTCGAGCTCCGTCGACAAGCTT

Protein Expression

Medium: InsectXpress medium (Lonza)
Antibiotics: Ampicillin
Procedure: Bacmid DNA was prepared from DH10Bac cells and using to transfect Sf9 insect cells for the preparation of initial baculovirus. AASS protein was expressed from infected Sf9 cells cultivated in InsectXpress medium (Lonza) for 72 hours at 27°C.

Protein Purification

Buffers: Binding Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4, 0.5 mM TCEP
Wash Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4, 0.5 mM TCEP
Elution Buffer: 50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Procedure: Harvested cells were resuspended in lysis buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 µL per 1 mL protease inhibitor cocktail EDTA-free).
Cell pellet was dissolved in approximately 200 mL lysis buffer and broken by homogenization by 2 passes at 12,000 psi. The cell debris was pelleted at 35000 x g, 1h and the supernatant used for purification on a gravity flow Ni-NTA column (5 mL).

The clarified cell extract was added to 5 ml of Ni-NTA resin pre-equilibrated with lysis buffer and passed through a glass column. The column was then washed with Binding Buffer (2 x 50 mL) and Wash Buffer (2 x 50 mL). The protein was eluted with Elution Buffer in 5 x 5 mL fractions. The eluted fractions from column 1 were pooled and concentrated to 5 mL with a 30 kDa MWCO spin concentrator and injected into an S200 16/60 column (pre-equilibrated in GF Buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol)) at 1.0 mL/min. 1.5 mL-fractions were collected. The eluted protein was cleaved overnight at 4 °C by TEV protease (1/20 (w/w)).
The following day protein sample was loaded onto 0.5ml Ni-sepharose column pre-equilibrated with GF buffer to remove uncleaved protein. Pooled protein fractions were concentrated to 13 mg/mL using a 30 kDa mwco concentrator.

Mass-spec Verification: yes
Compound Exact Mass: 687.106716

Structure Determination

Crystallization: NAD^+-bound crystals were prepared by mixing 100 nL of hAASS-SDH (18 mg/mL, in molar excess of NAD^+) with 50 nL of reservoir solution containing 25% PEG3350, 0.2M NaCl and 0.1M tris pH 8.5. Crystals were cryo-protected in 9% butanediol before freezing in liquid nitrogen.

Data Collection: *Beamline:* Dmnd I04; *Resolution:* 2.68 Å
Data Processing: The structure of hAASS-SDH was solved by molecular replacement with the program PHASER, using the fungal saccharopine reductase from *S.cerevisiae* (PDB code 2AXQ) as search model (38% sequence identity). Two molecules were found in the asymmetric unit (a.u.). The initial model was rebuilt using phenix.autobuild. A bound NAD^+ molecule in the active site was identified by difference Fourier method and manually placed into the electron density using Coot. To complete the model iterative cycles of phenix.refine including TLS refinement followed by manual model building for missing residues using coot were performed. No NCS restraints were applied due to conformational differences in domain III (res 278-376) of the two copies in the a.u. Solvent atoms were placed during the last four rounds of refinement using phenix.refine.