TAT

PDB:3DYD

Revision

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Entry Clone Accession:BC130534
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:ATTYA-k005
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfq*smWSVRPSDMAKKTFNPIRAIVDNMKVKPNPNKTMISLSIGDPTVFGNLPTDPEVTQAMKDALDS
GKYNGYAPSIGFLSSREEIASYYHCPEAPLEAKDVILTSGCSQAIDLCLAVLANPGQNILVPRPGFSLYKTLAESMGIEVKLYNLLP
EKSWEIDLKQLEYLIDEKTACLIVNNPSNPCGSVFSKRHLQKILAVAARQCVPILADEIYGDMVFSDCKYEPLATLSTDVPILSCGG
LAKRWLVPGWRLGWILIHDRRDIFGNEIRDGLVKLSQRILGPCTIVQGALKSILCRTPGEFYHNTLSFLKSNADLCYGALAAIPGLR
PVRPSGAMYLMVGIEMEHFPEFENDVEFTERLVAEQSVHCLPATCFEYPNFIRVVITVPEVMMLEACSRIQEFCEQHYHC

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 20 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 37 °C overnight. The overnight culture (20 ml) was used to inoculate two TunAir flasks with 0.75 l TB each, supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 0.1 ml Dow Corning anti-foam RD emulsion 63213 4D (BDH Silicone Products). The cultures were grown at 37 °C until OD600 reached ~2. The flasks were down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (38.8 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented with 2500 U Benzonase (Merck) and 1 tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure
Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)

Procedure
Purification of the protein was performed as a two step process on an ÄKTAxpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. The filtered lysate was loaded onto two HiTrap Chelating columns connected in series and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were pooled and fresh TCEP was added to a final concentration of 2 mM. The protein was subsequently concentrated using an Amicon Ultra-15 centrifugal filter device with 10,000 NMWL (Millipore) to 14.1 mg/ml in a volume of 0.7 ml and stored at -80 ºC. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,100 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. Pyridoxal-5´-phosphate was added to the protein solution (14 mg/ml) to a final concentration of 3 mM. 0.1 µl of the protein sample was then mixed with 0.1 µl of well solution consisting of 0.1 M sodium acetate, pH 4.6 and 30% MPD. The plate was incubated at 20 ºC and crystals appeared within 4 days. The crystal was flash-frozen in liquid nitrogen without cryo preservative.
NMR Spectroscopy:
Data Collection: Diffraction data to 2.3 Å resolution was collected at the ESRF beamline ID 14-2.
Data Processing: The structure was solved by molecular replacement with PHASER, using the structure of tyrosine aminotransferase from *Trypanosoma cruzi* (PDB entry 1BW0) as search model. The space group was C2 with cell dimensions a = 154.42 Å, b = 91.59 Å, c = 106.18 Å. Two monomers were located in the asymmetric unit. ARP/wARP was initially used for automatic model building. REFMAC5 was thereafter used for refinement and Coot for model building. Refinement using TLS-parameters was done at a later stage. Data in the interval 37.09 - 2.3 Å resolution were used, and after refinement the R values were R = 21.95% and Rfree = 26.09%. Coordinates were deposited in the Protein Data Bank, accession code 3DYD.