GNPNAT1-CoA-GlcNAc6P

PDB:2O28

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:GI:37620194
Entry Clone Source:MGC
SGC Clone Accession:
Tag:N-terminal: His-tag with integrated TEV protease site: MHHHHHHSSGRENLYFQ*G
Host:BL21 (DE3) codon plus RIL (Stratagene)

Construct

Prelude:
Sequence:

gMKPDETPMFDPSLLKEVDWSQNTATFSPAISPTHPGEGLVLRPLCTADLNRGFFKVLGQLTETGVVSPEQFMKSFEHMKKSGDYYV
TVVEDVTLGQIVATATLIIEHKFIHSCAKRGRVEDVVVSDECRGKQLGKLLLSTLTLLSKKLNCYKITLECLPQNVGFYKKFGYTVS
EENYMCRRFLK

Vector:p28-MHL

Growth

Medium:
Antibiotics:
Procedure:GNPNAT1 was expressed in E.coli BL21 (DE3) codon plus RIL in Terrific Broth (TB) in the presence of 50 µg/ml of kanamycin. Cell were grown at 37oC to an OD600 of 1.5 and induced by isopropyl-1-thio-D-galactopyranoside (IPTG), final concentration 1 mM, and incubated overnight at 15oC.

Purification

Procedure
The crude extract was cleared by centrifugation. The clarified lysate was loaded onto 5 ml HiTrap Chelating column (Amersham Biosciences), charged with Ni^{2+}. The column was washed with 10 CV of 20 mM HEPES, pH 7.4, containing 500 mM NaCl and 50 mM imidazole, 5% glycerol, and the protein was eluted with elution buffer (20 mM HEPES, pH 7.4, 500 mM NaCl, 250 mM imidazole, 5% glycerol). The protein was dialyzed against 20 mM HEPES, pH 7.4, 500 mM NaCl, 5% glycerol in the presence of TEV protease. The dialyzed protein was passed through a 5 ml Ni HiTrap column and loaded on Superdex200 column (26x60) (Amersham Biosciences), equilibrated with 20 mM PIPES buffer, pH 6.5, and 250 mM NaCl, at flow rate 4 ml/min. The

pooled fractions containing GNPNAT1 was further purified to homogeneity by ion-exchange chromatography on Source 30S column (10x10) (Amersham Biosciences), equilibrated with buffer 20 mM PIPES pH 6.5, and eluted with linear gradient of NaCl up to 0.5 M concentration (20CV). Purification yield was 25 mg of the protein per 1L of culture.

Extraction

Procedure
Cells were harvested by centrifugation at 7,000 rpm. The cell pellets were frozen in liquid nitrogen and stored at -80°C. For the purification the cell paste was thawed and resuspended in lysis buffer (50 mM HEPES, pH 7.4, 0.5 M NaCl, 5 mM imidazol, 2 mM ß-mercaptoethanol, 5% glycerol) with protease inhibitor (0.1 mM phenylmethyl sulfonyl fluoride, PMSF). The cells were lysed by passing through Microfluidizer (Microfluidics Corp.) at 20,000 psi.
Concentration:59 mg/ml
Ligand
MassSpec: Expected mass=20806.1 Da, measured mass =20831.1 Da
Crystallization: Purified GNPNAT1 was complexed with Coenzyme A and N-Acetyl-D-glucosamine-6-phosphate(Sigma) at 1:5 molar ratio of protein: CoA/ N-Acetyl-D-glucosamine-6-phosphate and crystallized using the hanging drop vapor diffusion method by mixing 2 microL of protein solution with 2 microL of the reservoir solution containing 27% PEG3350, 0.2 M MgCl2, 0.1 M Bicine, pH 9.0.
NMR Spectroscopy:
Data Collection:
Data Processing: