# GSG2 + K00224

**PDB:**2VUW

## Revision

**Revision Type:**created
**Revised by:**created
**Revision Date:**created
**Entry Clone Accession:**gi|56790919
**Entry Clone Source:**synthetic
**SGC Clone Accession:**
**Tag:**mhhhhhhssgvdlgtenlyfq*s(m) TEV-cleavable (*) N-terminal his6 tag.
**Host:**BL21 (DE3) phage resistant Rosetta strain

## Construct

**Prelude:**
**Sequence:**

mhhhhhhssgvdlgtenlyfq*sMGECSQKGPVPFSHCLPTEKLQRCEKIGEGVFGEVFQTIADHTPVAIKIIAIEGPDLVNGSHQK
TFEEILPEIIISKELSLLSGEVCNRTEGFIGLNSVHCVQGSYPPLLLKAWDHYNSTKGSANDRPDFFKDDQLFIVLEFEFGGIDLEQ
MRTKLSSLATAKSILHQLTASLAVAEASLRFEHRDLHWGNVLLKKTSLKKLHYTLNGKSSTIPSCGLQVSIIDYTLSRLERDGIVVF
CDVSMDEDLFTGDGDYQFDIYRLMKKENNNRWGEYHPYSNVLWLHYLTDKMLKQMTFKTKCNTPAMKQIKRKIQEFHRTMLNFSSAT
DLLCQHSLFK

**Vector:**pNIC28-Bsa4

## Growth

**Medium:**
**Antibiotics:**
**Procedure:**1ml from a 10 ml overnight culture containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol was used to inoculate 1 liter of LB media containing the same concentration of antibiotics. Cultures were grown at 37°C until the OD600 reached ~0.3. After that the temperature was adjusted to 20°C. Expression was induced over night using 1mM IPTG at an OD600 of 0.8. For Selenomethionine labelling, 1ml from a 10 ml overnight culture containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol was grown at 37°C overnight. The cells then were washed and cultured in 12L MD media with 40mg of Selenomethionine/L at 37°C. When the OD reached 1.0, 0.5 mM (final concentration) of IPTG was added and the temperature was decreased to 18°C for overnight culture. The cells were collected by centrifugation and the pellet resuspended in binding buffer and frozen. Binding buffer: 50mM HEPES pH 7.5; 500 mM NaCl; 5% glycerol, 10 mM imidazole.

## Purification

**Procedure**

Column 1: Ni-affinity chromatography.Buffers: Binding buffer: Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycero, 10mM Imidazole. Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol. Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM imidazole, 5% Glycerol.Procedure: 5 ml of 50% Ni-NTA slurry (Qiagen) was applied to a 1.5 x 10 cm gravity column. The column was equilibrated with 50 ml binding buffer. The lysate was applied to the column which was subsequently washed with 50 ml wash buffer 1 and 2. The protein was eluted by gravity flow by applying 5 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150mM, 250 mM); fractions were collected until essentially all protein was eluted. The eluted protein was analyzed by SDS-PAGE. DTT was added to the protein sample to a final concentration of 5mM.Column 2: Size exclusion chromatography (Superdex S75, 60 x 1cm)SEC-Buffers: 50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM DTT.Procedure: The fractions eluted of the Ni-affinity chromatography were concentrated to about 4 mls using Centricon concentrators (10kDa cut off). The concentrated protein was applied to a Superdex S75 column equilibrated in SEC buffer at a flow rate of 0.8 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

## Extraction

**Procedure**
Cell pellets were lysed by C5 high pressure homogenizer (Avestin). The lysate was centrifuged at 21,000 rpm for 60 minutes and the supernatant collected for purification.
**Concentration:**Centricon with a 30 kDa cut off in SEC-buffer.
**Ligand**
**MassSpec:**The mass of the protein was calculated to be 40655 Da and experimentally determined mass was 40663 Da for the His tag containing protein. The expected mass of Selenomethionine labelled protein is 41,077 and experimental mass for fully labelled protein is 41,086. The identity of the protein was reconfirmed to be correct by DNA sequencing both DNA strands of this expression construct.
**Crystallization:**All crystallizations were carried out using sitting drop vapour diffusion at 4°C. Crystallization of protein was performed by matrix-seeding with crushed native crystals into 200nl drops composed of equal volumes of native protein (10 mg/ml) with the ligand, 5-Iodotubercidin; 4-Amino-5-iodo-7-(b-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (1mM) and reservoir solution containing: 0.1M SPG pH 7.0; 60.0% MPD. Seeding crystals obtained from 100nl drops composed of equal volume of protein (14mg/ml) and reservoir solution containing: 0.1M MMT pH 6.0; 60.0% MPD.Crystallization of SeMet-substituted protein was performed by matrix-seeding with crushed native crystals into 200nl drops composed of equal volumes of SeMet protein (8mg/ml) with the ligand, 5-Iodotubercidin; 4-Amino-5-iodo-7-(b-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (1mM)and reservoir solution (0.15M NH4cit pH5; 25% PEG3350; 0.1M MES pH6.0).Crystals of the ATP complex were grown in using a reservoir solution containing 0.2M Li2SO4; 0.1M HEPES pH 7.5; 25% PEG 3350. Crystals were flash frozen in liquid nitrogen using the same solution and 25% ethylene glycol.
**NMR Spectroscopy:**
**Data Collection:** Crystals were cryo-protected using the well solution supplemented with additional MPD and flash frozen in liquid nitrogen. Data were collected at beamline X10SA at the Swiss Light Source (SLS).
**Data Processing:**Initial phases were determined using SAD data collected from crystals of selenomethionine-substituted protein at the Se peak ($\lambda = 0.9792$Å) to a resolution of 2.2 Å. Se sites were located with SHELXD, and phases were calculated with SHARP, with subsequent density modification by DM. The model was built using the Buccaneer program into this map, and completed manually in COOT. This model was used as a molecular replacement search model against another dataset from Se-Met substituted protein containing 5-Iodotubercidin

collected at below the Se-edge ($\lambda$ =0.97640 Å) and refined against data to 1.8 Å to a final Rfree of 17.8%.