RASEF

PDB:2PMY

Revision

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Entry Clone Accession:gi:23270807
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SGC Clone Accession:HPC046A1
Tag:N-terminal hexahistidine tag
Host:E.coli. BL21 (DE3) codon(+) RIL

Construct

Prelude:
Sequence:

gADGDGEELARLRSVFAACDANRSGRLEREEFRALCTELRVRPADAEAVFQRLDADRDGAITFQEFARGFLGSL

Vector:p28a-tev-mhl

Growth

Medium:TB
Antibiotics:
Procedure: We prepared the seeds by inoculating glycerol stock of E. coli cells BL21-CodonPlus (DE-3)-RIL into 100 mL of Luria-Bertani medium. After overnight growth, all of the seeds were inoculated into 3.6 L of Terrific Broth medium in the presence of 50 µg/mL of kanamycin and 50 µg/mL chloramphenicol at 37 ºC and grown to an OD600 between 3-5. Cells were then induced by isopropyl-1-thio-D-galactopyranoside at the final concentration of 1.0 mM and grown overnight at 18ºC in the SGC LEX bubbling system.

Purification

Procedure
Column 1: Ni-NTA beads
Column 2: Size exclusion chromatography (Superdex 75 26/60)
1 ml of Ni-NTA suspension solution was added into 40 ml cell lysis supernatant solution. The mixture was shaken for 1.5 hours at 4 ºC. Beads were collected with centrifuge at 2500 rpm, 5 minutes. Beads were washed with 25 ml washing buffer, then collected with centrifuge. Protein was eluted with 15 ml elution buffer. TEV was added into the eluted protein solution, and the solution was shaken at 4 ºC overnight to remove the His tag. Incubated solution was reloaded into Ni-NTA column to remove the TEV protease and uncut protein.

The fractions purified with the Ni-affinity chromatography applied to a Superdex S75 column equilibrated in SEC buffer at a flow rate of 2.0 ml/min. Eluted fractions were 95% pure as judged by SDS-PAGE.

Extraction

Procedure
Cultures were centrifuged and the cell pellets were harvested and stored at -80 ºC before use. Cells were thawed and suspended in 200 mL binding buffer with 0.5% CHAPS (Sigma) and 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.5% (v/v) protease inhibitor cocktail (Sigma), 1 mM Benzamidine, 1600 units Benzonase (Sigma), 1 mM TCEP and lysed with Avestin C5 biotech processor. The lysate was centrifuged at 16000 rpm for 45 min and the supernatant was used for subsequent steps of purification. All the extraction steps were carried out at 4 ºC.
Concentration: Protein concentrated to 19 mg/ml Concentrator with a 5 kDa cut off in SEC-buffer.
Ligand
MassSpec:
Crystallization: Crystals were obtained using the vapor diffusion method and a protein concentration of 19 mg/ml. 2 µl of the concentrated protein mixed with 2 µl of a well solution containing 0.7 M Ammonium Sulfate, 0.1 M Na Cacodylate, pH 5.0, 0.01 M MgCl2, 10 % Glycerol. Crystals appeared after one day at 18°C.
NMR Spectroscopy:
Data Collection: Crystals were cryo-protected using the mixture of mother liquor and glycerol, and flash frozen in liquid nitrogen. Diffraction data were collected at the home source to 2.3 Å.
Data Processing: