USP5

PDB:3IHP

Revision

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Tag:USP5 N-terminal tag: MGSSHHHHHHSSGLVPRGS
Host:BL21 (DE3)

Construct

Prelude:
Sequence:

Usp5.001.835:

mgsshhhhhhssglvprgsMAELSEEALLSVLPTIRVPKAGDRVHKDECAFSFDTPESEGGLYICM
NTFLGFGKQYVERHFNKTGQRVYLHLRRTRRPKEEDPATGTGDPPRKKPTRLAIGVEGGF
DLSEEKFELDEDVKIVILPDYLEIARDGLGGLPDIVRDRVTSAVEALLSADSASRKQEVQA
WDGEVRQVSKHAFSLKQLDNPARIPPCGWKCSKCDMRENLWLNLTDGSILCGRRYFDG
SGGNNHAVEHYRETGYPLAVKLGTITPDGADVYSYDEDDMVLDPSLAEHLSHFGIDMLK
MQKTDKTMTELEIDMNQRIGEWELIQESGVPLKPLFGPGYTGIRNLGNSCYLNSVVQVL
FSIPDFQRKYVDKLEKIFQNAPTDPTQDFSTQVAKLGHGLLSGEYSKPVPESGDGERVPEQ
KEVQDGIAPRMFKALIGKGHPEFSTNRQQDAQEFFLHLINMVERNCRSSENPNEVFRFLV
EEKIKCLATEKVKYTQRVDYIMQLPVPMDAALNKEELLEYEEKKRQAEEEKMALPELVR
AQVPFSSCLEAYGAPEQVDDFWSTALQAKSVAVKTTRFASFPDYLVIQIKKFTFGLDWVP
KKLDVSIEMPEELDISQLRGTGLQPGEEELPDIAPPLVTPDEPKAPMLDESVIIQLVEMGFP
MDACRKAVYYTGNSGAEAAMNWVMSHMDDPDFANPLILPGSSGPGSTSAAADPPPEDC
VTTIVSMGFSRDQALKALRATNNSLERAVDWIFSHIDDLDAEAAMDISEGRSAADSISESV
PVGPKVRDGPGKYQLFAFISHMGTSTMCGHYVCHIKKEGRWVIYNDQKVCASEKPPKD
LGYIYFYQRVAS

Ubiquitin.01.75:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQ

KESTLHLVLRLRG
Vector:pET28a-LIC for USP5; pTYB2 for UBH

Growth

Medium:TB
Antibiotics:
Procedure:For USP5: Competent BL21 (DE3) cells (Invitrogen C6000-03) were transformed and grown using the LEX system (Harbinger BEC) at 37 degC in 2L bottles (VWR 89000-242) containing 1800 ml of TB (Sigma T0918) supplemented with 150 mM glycerol, 100 µg/ml Kanamycin and 600 µl antifoam 204 (Sigma A-8311). When OD600 about 6 was reached, the temperature was reduced to 15 degC, and one hour later protein expression was induced with 100 µM IPTG (BioShop IPT001) and the culture was incubated overnight (16 hours) at 15 degC. Cell pellets were collected by centrifugation (12,227 x g, 20 min), frozen and stored at -80 degC.

For UBH: Competent BL21 (DE3) cells were transformed with the Ubiquitin plasmid and grown as above in TB medium supplemented with 100 µg/ml ampicillin instead of Kanamycin.

Purification

Procedure
For USP5: The cleared lysate was loaded onto a 3 mL TALON metal-affinity resin column (BD Biosciences) at 4ºC. The column was washed with 10 mL Wash Buffer A, 10 mL Wash Buffer B and 10 mL Wash Buffer A. The protein was eluted with 6 mL Elution Buffer. The N-terminal His-tag was removed by overnight incubation of the protein with thrombin (1 unit/mg protein) at 4°C. The protein was further purified by gel filtration on a HighLoad 16/60 Superdex 200 column (GE Healthcare) using Gel Filtration Buffer. Fractions containing protein corresponding to the Usp5 peak were pooled and concentrated by ultrafiltration. The yield of the protein was 5 mg per liter of bacterial culture.

For UBH protein purification: The clarified lysate was mixed with chitin beads New England Biolabs S6651L) equilibrated with the Lysis Buffer (2.5 ml beads per 50 ml lysate). The suspension was incubated with constant stirring for 2 h at 37 degC and poured into an open-end column. The column was drained and the beads were washed with 10 x volume Lysis Buffer. The column was then filled with 1 vol. of 100 mM 2-mercaptoethanesulfonic acid (MESNA, Sigma M1511), closed tightly, gently shaken to resuspend the resin in the MESNA solution, plugged and left overnight (16 h) at room temperature. The column was then opened and the eluate was collected and combined with additional washing of the column (2 vol.) with the MESNA solution. The combined eluate, containing ubiquitin(1-75)-MESNA thiol ester, was concentrated to a final volume of ~ 5 ml and loaded onto a HighLoad 16/60 Superdex 200 column equilibrated with the Lysis Buffer. The gel-filtration fractions were collected, analyzed by LC/MC and those containing purified ubiquitin(1-75)-MESNA thiol ester were combined, divided into 1-ml aliquots 10-fold, frozen with liquid nitrogen and stored at -80 degC. The yield of the thiol ester

was approx. 7 mg per liter bacterial culture.

Preparation of Suicide Substrate: The suicide substrate, ubiquitin(1-75)-bromoethylamide, was prepared using a scaled-up and modified protocol by Wilkinson et al.[4]. In order to prepare ubiquitin(1-75)-bromoethylamide, 150 mg 2-bromoethylamine hydrobromide (Sigma B65705) was dissolved in 1 ml ubiquitin(1-75)-MESNA thiol ester solution (3 mg/ml) and 200 microL 2 M NaOH was added. The reaction mixture was incubated for 20 min at room temperature and dialyzed against 4 l Dialysis Buffer (20 mM sodium acetate, pH 5.5) for 2 h at 4 degC using a Slide-A-Lyzer with molecular-weight cut-off limit of 3,500 (Pierce). The pH of the dialyzed solution was adjusted to 7.5, NaCl was added to it to a final concentration of 0.5 M and it was immediately used for Usp5 modification.

Modification with Suicide Substrate: A 32 mg amount of Usp5 was incubated with 10-fold molar excess of ubiquitin(1-75)-bromoethylamide for 1 h at room temperature (21°C). The covalent complex was purified by gel-filtration chromatography as Usp5 above and concentrated by ultrafiltration to a final concentration of 25 mg/ml. The concentrated protein was stored on ice. Coomassie-stained SDS-PAGE showed that the product was pure and analysis by LC/MS (Agilent 1100 Series) showed that its molecular weight corresponded to the calculated molecular weight of the Usp5 construct, with N-terminal His-tag removed, covalently linked to ubiquitin 1-75 through an ethylene spacer.

Extraction

Procedure
For USP5: After resuspension in 30 mL per liter bacterial culture of Lysis Buffer, cells were lysed using a Microfluidics M110-EH microfluidizer at 18,000 psi.

For UBH protein: The bacterial cells from 1 l culture were suspended in 50 ml Ub-MESNA Lysis Buffer and cells were lysed using a Microfluidics M110-EH microfluidizer at 18,000 psi. Cell debris was removed by centrifugation at 20,000g for 30 min.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals of the covalent ubiquitin complex of Usp5 were grown at 298 K using the hanging drop method by mixing equal volumes of protein solution (25 mg/ml) and Crystallization Buffer (1.45 M ammonium sulfate, 0.1 M bis-Tris, pH 6.5, 0.2 M sodium acetate, 5% ethyleneglycol and 1 mM dithiothreitol). The crystals were cryoprotected by immersion in Paratone N in paraffin oil 30% (v/v) and placed in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data from a crystal of the Usp5-ubiquitin complex were collected at beamline 23-ID-B of the GM/CA-CAT at the Advanced Photon Source (Argonne National Laboratory). The dataset was integrated and scaled using the HKL program suite.
Data Processing: With the aid of the computer program PHASER, a partial molecular replacement solution was found using as search models the structures of the covalent ubiquitin-USP2 complex (PDB code 2IBI) and the USP5 ZnF UBP domain (PDB code 2G43). Truncated NMR models of the first (PDB code 2DAG) and second (2DAK) UBA domains of USP5 were positioned into difference electron density as the phases improved with iterative manual model building using the graphics program Coot, and TLS and restrained refinement using REFMAC5.

The model of the cryptic N-terminal Znf-UBP was built at the later stages of refinement. Due to the relatively low resolution of this structure, NCS restraints were used throughout the refinement. Initial TLS parameters were obtained from the TLSMD web server. The final model, comprising 11765 protein atoms, 2 Zn, 1 Cl and 32 solvent atoms, has been refined to an Rfactor of 0.224 (Rfree 0.276) for data from 34.9 to 2.8 angstroms.