



Deducing the Crystal Structure of MERS-CoV Helicase

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Abstract

RNA virus encodes a helicase essential for viral RNA transcription and replication when the genome size is larger than 7 kb. Coronavirus (CoV) has an exceptionally large RNA genome (~30 kb) and it encodes an essential replicase, the nonstructural protein 13 (nsp13), a member of superfamily 1 helicases. Nsp13 is among the evolutionary most conserved proteins not only in CoVs but also in nidovirales. Thus, it is considered as an important drug target. However, the high-resolution structure of CoV nsp13 remained unavailable even until more than a decade after the outbreak of the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003, which hindered the structure-based drug design. This is in part due to the intrinsic flexibility of nsp13. Here, we describe protocols of deducing the crystal structure of Middle East respiratory syndrome coronavirus (MERS-CoV) helicase in detail, which include protein expression, purification, crystallization, enzymatic characterization, and structure determination. With these methods, catalytically active recombinant MERS-CoV nsp13 protein can be prepared and crystallized and the crystal structure can be solved.

Key words Coronavirus, Helicase, nsp13, Crystallization, Structure determination

1 Introduction

Coronavirus (CoV) remains a public health concern 16 years after the outbreak of the severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 [1]. The Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in 2012, reemerged in 2015, and is still circulating in the Middle East region, which reminds the international community that the threat of CoVs persists [2, 3]. However, neither vaccine nor drugs against CoVs are currently available. Outbreaks of CoVs initiated extensive structural investigation on CoV encoded proteins thereafter, which not only shed light on the life cycle of CoVs but also laid foundation for the structure-based drug design (SBDD). CoV contains a positive single-stranded RNA genome of ~30 kb, one of the largest among +RNA viruses [4, 5]. To maintain the unusually large RNA genome, CoV encodes two replicase polyproteins pp1a and pp1ab, which are broken down into 16 nonstructural proteins

(nsps) via proteinase cleavage [6, 7]. The nsps are then recruited to cytoplasm membranes, on which they form the membrane-associated replication-transcription complex (RTC). An RNA-dependent RNA polymerase nsp12 and a helicase nsp13 are the central components of RTC [8, 9]. However, while high-resolution structures of most CoV encoded proteins had been determined soon after SARS-CoV outbreak, the first CoV nsp13 structure, MERS-CoV nsp13, was only solved recently [10]. Nsp13 belongs to helicase superfamily 1 and shares conserved features with the eukaryotic Upf1 helicase [11, 12]. Nsp13 is a multi-domain protein comprising of an N-terminal Cys/His rich domain (CH domain) and a C-terminal SF1 helicase core [10]. Nsp13 exhibits multiple enzymatic activities, including hydrolysis of NTPs and dNTPs, unwinding of DNA and RNA duplexes with 5'-3' directionality and the RNA 5'-triphosphatase activity [13, 14]. To investigate the structure of CoV nsp13, we overexpressed the full-length MERS-CoV nsp13 (1-598aa) in insect cells and purified. The activity of the recombinant MERS-CoV nsp13 was verified by ATPase and helicase assays. Crystallization of MERS-CoV nsp13 was achieved by adding a synthetic single-stranded 15 poly dT DNA with 5'-triphosphate (ppp-15 T) to the protein, which restrains the intrinsic flexibility of nsp13. Benefiting from the presence of an N-terminal zinc-binding domain with three zinc atoms, multi-wavelength anomalous diffraction (MAD) data at the zinc absorption edge was collected, which allowed the determination of the crystal structure of MERS-CoV nsp13 [10].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω -cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to reagents.

2.1 Gene Cloning

1. Full-length MERS-CoV (GenBank accession: YP_009047202) nsp13 gene cDNA (GenScript).
2. The forward primer (gaaattggaatccgctgtcggttcatgc) and the reverse primer (gaaattctcgagtcactggagcttgaatt) of full-length nsp13. Primers stocks are either supplied or diluted by molecular biology grade water to 100 μ M and stored at -20 °C.
3. The pFastbac-1 baculovirus transfer vector is modified; 6 \times Histidine-SUMO tag with a C terminal PreScission protease (PPase) site coding sequence in the N terminal of open reading frame [15].

4. Chemically competent bacterial cells of *E. coli* BL21 and *E. coli* DH10 Bac are prepared in-house as described.
5. High-fidelity PCR master mix with HF buffer (2 × fusion).
6. Endonuclease *Bam*HI and *Xho*I (fast digest).
7. 50× TAE buffer (1 L): 242 g Tris base, 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA, pH 8.0, pH adjusted to 8.5. Filtered through a 0.2 μm membrane filter and used as a 1× solution.
8. Rapid DNA ligation kit (Promega).
9. LB medium: 5 g yeast extract, 10 g tryptone, and 10 g NaCl are dissolved in 800 mL water. Volume is adjusted to 1000 mL and autoclaved on the same day.
10. LB-agar: 5 g yeast extract, 10 g tryptone, 10 g NaCl, and 15 g agar are dissolved in 800 mL water. Volume is adjusted to 1000 mL and autoclaved on the same day.
11. 1000× Antibiotic stocks: Ampicillin (100 mg/mL); Kanamycin (50 mg/mL); Tetracycline (10 mg/mL in ethanol); Gentamycin (7 mg/mL), stored at −20 °C. All stocks prepared in water are filtered through a 0.22 μm syringe filter.

2.2 Bac-to-Bac Baculovirus Expression

1. Blue-gal (100 mg/mL in DMSO); IPTG (40 mg/mL) stored at −20 °C. All stocks prepared in water are filtered through a 0.22 and 0.45 μm syringe filter.
2. Bacmid transfection reagent.
3. Bacmid extract kit (plasmid mini kit (100)) (Qiagen).
4. Insect cell: Sf21 and High-5 (Invitrogen).
5. Insect cell media: Sf900II medium and High express five (Invitrogen); SIM HF (Sino Biological Inc).
6. 75 cm² flasks.

2.3 Test Expression

1. Lysis and wash buffer (I): 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 20 mM imidazole.
2. Elution buffer: 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 250 mM imidazole.
3. Ni-IDA Metal Chelate Resin (Qiagen).

2.4 Large-Scale Expression and Purification

1. Lysis and wash buffer(II): 25 mM Tris–HCl, pH 7.5, 1.5 M NaCl, 20 mM imidazole.
2. PreScission protease (PPase) was prepared in-house.
3. Econo-Columns.
4. Amicon Ultra protein concentrators (Millipore).

5. Size Exclusion Chromatography (SEC) buffer: 10 mM HEPES, pH 7.0, 100 mM NaCl. Filtered through a 0.2 μ m membrane filter and stored at 4 °C.
6. Size-exclusion chromatography (Superdex-200) (GE health-care).

2.5 ATPase Assay

1. [γ -³²P]ATP.
2. ATP is dissolved by water to 1 mM.
3. 5 \times ATPase reaction buffer: 20 mM MgCl₂, 500 mM Tris-HCl, pH 8.0.
4. The thin-layer chromatography cellulose (TLC) plates.
5. ATPase reaction quenching buffer: 0.5 M EDTA.
6. TCL plates running buffer: 0.8 M acetic acid and 0.8 M LiCl.
7. Typhoon Trio Variable Mode Imager.

2.6 Helicase Assay

1. ATP, GTP, CTP, and TTP.
2. Trap RNA (5'-CGAAGCUGCUAACAUCAG-3'), top strand RNA (5'-UUUUUUUUUCUGAUGUUAGCAGCUUCG-3'), and bottom stand RNA with a 5'-HEX tag (5'-HEX-CGAAGCUGCUAACAUCAG-3').
3. The partial duplex RNA substrate with 5' overhang is prepared by mixing the top strand RNA and the 5'-HEX tagged bottom strand.
4. 10 \times Helicase reaction buffer: 500 mM Hepes, pH 7.5, 50 mM MgCl₂, 20 mM DTT, and 0.1% BSA.
5. 5 \times loading buffer: 100 mM Tris-HCl, pH 7.5, 50% glycerol, and 1% SDS.
6. 10 \times TBE buffer: 108 g Tris, 7.44 g Na₂EDTA.2H₂O, 55 g boric acid, dissolved by water and adjusted the volume to 1 L.
7. 6% native PAGE gel: 1 mL 10 \times TBE, 5.2 mL 30% polyacrylamide, 0.39 mL glycerol (50%), 13.25 mL H₂O, 0.15 mL ammonium persulfate (10%), and 0.01 mL TEMED.

2.7 Crystallization

1. Crystallization screen kits (Hampton and Qiagen).
2. 5'-triphosphate DNA (ppp-15T) are synthesized and purified according to previously published procedures [16, 17].
3. 24-Well vapor diffusion crystallization plates.
4. Crystallization conditions screen kits are supplied by Hampton research.

2.8 Structure Determination

The software that are used in structure determination include XDS, Coot, SHARP/autoSHARP, PHENIX, and Pymol.

3 Methods

All procedures should be carried out at room temperature unless otherwise specified.

3.1 Transfer Plasmid Construction and Transposition in *E. coli* DH10 Bac

1. Amplify MERS-nsp13 full-length by PCR method with BamHI and XhoI restriction sites at 5' and 3' termini, respectively.
2. The amplified MERS-nsp13 gene should be digested by BamHI/XhoI at 37 °C for 1 h. The pFast-bac-6×Histidine-SUMO plasmid should also be digested by BamHI/XhoI at the same time.
3. Digested nsp13 DNA should be ligated with pFast-bac-6×Histidine-SUMO vector using the rapid DNA ligation kit. The ligation system: 100 ng DNA, 35 ng vector, 10 × reaction buffer and T4 ligase, mixed and incubated at room temperature for 2 h.
4. Add 10 µL mixture of ligation product to 100 µL *E. coli* BL21 competent cells in a 1.5 mL Eppendorf tube and incubate on ice for 30 min. Heat shock the cells for 90 s in a 42 °C water bath and return briefly to ice. After 2 min, add 300 µL LB medium and incubate in a shaker at 37 °C and shake at 200 rpm for 1 h.
5. Spread the culture onto the LB plate containing Ampicillin (100 µg/mL), and incubate at 37 °C for 14–16 h.
6. Pick single colonies and inoculate to 500 µL LB medium. Incubate cultures at 37 °C and shake at 200 rpm for 4 h. Send the cultures for sequencing.
7. Collect the positive colonies and extract the recombinant plasmids according to the sequencing result.
8. Add 20 ng recombinant plasmid to 50 µL *E. coli* DH10 Bac competent cells in a 1.5 mL Eppendorf tube and incubate on ice for 30 min. Heat shock the cells for 90 s in a 42 °C water bath and return briefly to ice. 2 min later, add 500 µL LB medium and incubate in a shaker at 37 °C and shake at 200 rpm for 5 h.
9. Prepare bacmid selection plates containing approximately 10 mL of LB-agar, supplemented with 50 µg/mL kanamycin, 7 µg/mL gentamycin, 10 µg/mL tetracycline, 40 µg/mL IPTG, and 100 µg/mL Blue-gal, and once set allow to dry, inverted at room temperature.
10. Spread 50 µL culture onto the bacmid selection plate and incubate at 37 °C for up to 60 h.

3.2 Production of MERS-CoV nsp13 bacmid

1. Pick single white colony from the bacmid selection plate (white colonies contain the recombinant bacmid DNA and the blue ones do not). Inoculate it to 15 mL LB (containing 50 µg/mL kanamycin, 7 µg/mL gentamycin, 10 µg/mL tetracycline) and incubate at 37 °C with shaking at 220 rpm for up to 5 h.
2. Centrifuge the culture at $3000 \times g$ for 20 min. Remove the supernatant carefully. Add 1.2 mL solution P1 from the bacmid extract kit (plasmid mini kit (100) from Qiagen) and resuspend the precipitate.
3. Add 1.2 mL solution P2, mix thoroughly by softly inverting 6–8 times, and incubate at room temperature (about 25 °C) for 5 min.
4. Add 1.2 mL solution P3, mix thoroughly by softly inverting 6–8 times. Incubate it on ice for 5 min.
5. Centrifuge at $15,000 \times g$ for 10 min at 4 °C. Apply the supernatant to the QIAGEN-tip and allow it to enter the resin by gravity flow.
6. Wash the QIAGEN-tip with 2×2 mL Buffer QC. Elute DNA with 0.8 mL Buffer QF into a clean 1.5 mL Eppendorf tube.
7. Precipitate DNA with 0.56 mL isopropanol and wash the pellet with 1 mL 70% ethanol. Dry the pellet and resuspend in 20 µL sterilized ddH₂O.

3.3 Production of MERS-CoV nsp13 Recombinant Virus

1. Seed 0.8×10^6 Sf21 cells in duplicates in 6-well plates and incubate for 15–30 min at 28 °C (*see Notes 1 and 2*).
2. Prepare transfection reagent solution of 100 µL SF 900 II media (nonantibiotic) with 6 µL transfection reagents. Prepare bacmid solution of 100 µL SF 900 II media (nonantibiotic) with 4 µL dissolved bacmid. Mix the two solutions and rest at 28 °C for 30 min.
3. Add 800 µL SF 900 II media (nonantibiotic) to the mixture, and transfer them to the 6-well plates in **step1** (after removing supernatant and washed by nonantibiotic media twice). Prior to addition of SF90II media to the 6-well plates make sure to remove the supernatant and wash twice with nonantibiotic media. Incubate the 6-well plate at 28 °C for 5 h.
4. Remove the supernatant of the plates and add 2.5 mL fresh media with 10 µg/mL gentamycin. Incubate at 28 °C for 72 h. Observe the cells under the microscope. Collect the supernatant if the cells present noticeable infected symptoms (swelling, splitting, and stop growing), and centrifuge at $453 \times g$ for 5 min. Collect the supernatant. This will be P1 virus.
5. Prepare 75 cm² flasks containing 15 mL Sf21 cell suspension at a density of $0.4\text{--}0.6 \times 10^6$ cells/mL. Make sure the cells are distributed evenly in the flasks, incubate at 28 °C for 20 min.

6. Add 0.4 mL P1 virus to the 15 mL Sf21 cells culture in 75 cm² flask. Incubate at 28 °C for 48–60 h. Observe the cells under the microscope and collect the supernatant from the flasks, centrifuge at 453 × *g* for 5 min. Collect the supernatant. This will be P2 virus.
7. Prepare 75 cm² flasks containing 15 mL Sf21 cells suspension at a density of 0.6–1.0 × 10⁶ cells/mL. Make sure the cells are distributed evenly in the flask, incubate at 28 °C for 20 min.
8. Infect the 15 mL Sf21 cell culture in 75 cm² flask with 0.4 mL P1 virus. Incubate at 28 °C for 48–60 h. Observe the cells under the microscope and collect the supernatant from the flasks, centrifuge at 453 × *g* for 5 min. Collect the supernatant (P3 virus).

3.4 Test Expression of MERS-CoV nsp13

1. Prepare 50 mL high-5 cells in express-5 medium at a density of 0.38 × 10⁶ cells/mL, and culture in a 300 mL cell conical flask. Incubate the culture at 28 °C with shaking at 120 rpm for 48 h, the density of cells will grow to 1.5–2.5 × 10⁶ cells/mL (*see Note 2*).
2. Add 1.5 mL MERS-CoV nsp13 P2 or P3 virus into the culture, and incubate at 22 °C with shaking at 120 rpm for 44–60 h.
3. Centrifuge the culture at 3000 × *g* for 30 min. Collect the cells pellet.
4. Quickly freeze the cells pellet by liquid nitrogen. Resuspend the pellet by 5 mL lysis and wash buffer (I) and incubate on ice for up to 10 min.
5. Centrifuge at 15000 × *g* for 20 min at 4 °C. Apply the supernatant to the mini-affinity column with 300 μL Ni-IDA Metal Chelate Resin and allow it to enter the resin by gravity flow at 4 °C.
6. Wash the resin with 1 mL lysis and wash buffer (I) three times.
7. Load 600 μL elution buffer to the resin and allow it to enter the resin by gravity flow. Collect the eluted sample into a 1.5 mL Eppendorf tube.
8. Pick 8 μL eluted sample and mix with 2 × loading buffer.
9. Load SDS-PAGE gel and run at 200 V for 60 min. The result of expression is visualized by Coomassie brilliant blue stain (Fig. 1).

3.5 Large-Scale Expression and Purification of MERS-CoV nsp13

1. Prepare 1 to 1.5 L high-5 cells in HF medium at a density of 0.38 × 10⁶ cells/mL, and culture in 3 L cell conical flask (keep the volume of culture to 500–750 mL in a 3 L flask). Incubate the culture at 28 °C with shaking at 120 rpm for 40–48 h, then the density of cells will grow to 1.5–2.5 × 10⁶ cells/mL (*see Note 3*).

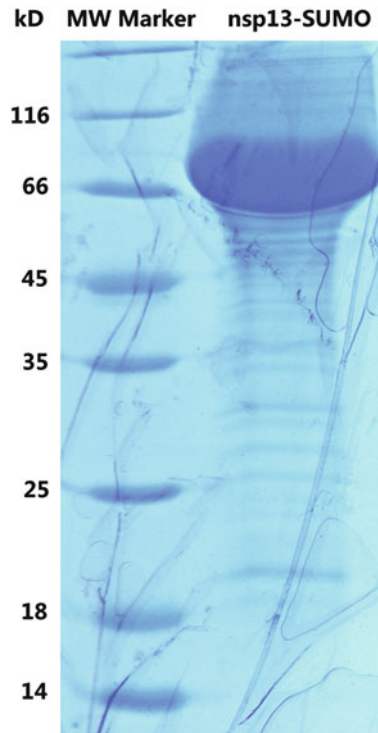


Fig. 1 Test expression of MERS-CoV nsp13. The eluted MERS-CoV nsp13 protein possesses an 6-Histidine and SUMO tag. 8 μ L sample was loaded on the SDS-PAGE gel and the result was visualized by Coomassie brilliant blue stain

2. Add 30–45 mL MERS-CoV nsp13 P3 virus to the culture (30 mL virus/L culture). Incubate the culture at 22 °C with shaking at 120 rpm for 40–48 h.
3. Centrifuge the culture at $3000 \times g$ for 30 min. Collect the cell pellet.
4. Resuspend the pellet by 100–150 mL lysis and wash buffer (II) (100 mL buffer/1 L culture's pellet). Add 600–900 μ L 0.1 M PMSF to the cell suspension (600 μ L PMSF/100 mL cell suspension).
5. Place the cell suspension on ice-water mixture. Set the amplitude to 30% on a 750 W cell sonicator and sonicate with bursts of 3 s on, 5 s off.
6. Transfer the lysates to centrifuge tubes, balance the tubes pairwise and centrifuge at $15000 \times g$ for 1 h at 4 °C.
7. Transfer the supernatant to new centrifuge tubes and re-centrifuge at $15,000 \times g$ for 1 h at 4 °C.
8. Transfer the clear supernatant into clean tubes taking care to avoid transferring any pelleted material.
9. Filter the supernatant by 0.45 μ m syringe filter. This clarified supernatant represents the soluble fraction.

10. Prepare the Ni-NTA resin, and add the resin into 2–3 empty Econo-Columns (5 mL 50% resin per column), wash and balance the resin with 10 mL lysis and wash buffer(II) twice.
11. Place the columns at 4°C. Apply the clarified cell lysates supernatant to the balanced Ni-NTA resin, and flow through the column by gravity.
12. Wash the resin in the column with 10 mL lysis and wash buffer (II) 3 times.
13. Resuspend the resin by 3.5 mL L lysis and wash buffer(II), and add 100 μ L PPase. Incubate the resin at 4 °C for 10–12 h.
14. Apply the buffer to the column and let it flow under gravity. Collect the flow through in a 50 mL tube.
15. Add another 25 mL lysis and wash buffer(II) to the resin and flow through the column. Also collect the flow through in the previous 50 mL tube.
16. To remove the PPase, add the flow through to another column which contains the NS4B resin. Collect the flow through from the NS4B resin column.
17. Apply the flow through to an Amicon Ultra protein concentrator (30 kDa filter, 50 mL), centrifuge at $2465 \times g$ at 4 °C until the sample volume is concentrated to 1 mL.
18. Transfer the concentrated sample to a 1.5 mL tube and centrifuge at $17,949 \times g$ for 3 min to remove the aggregates and particulates.
19. Load the sample onto the superdex 200 column in the size exclude chromatography (SEC) buffer using an ÄKTA-purify chromatography at 4 °C.
20. Analyze 8 μ L of each peak fractions by SDS-PAGE (Fig. 2).
21. Collect the fractions that contain the single band of MERS-CoV nsp13, mix the fractions, and concentrate the mixture to a final density of 6–8 mg/mL.
22. 50 μ L packaged the protein sample, quickly freeze them by liquid nitrogen and store them at -80 °C.

3.6 ATPase Assay of MERS-CoV nsp13

1. Dilute the purified MERS-CoV nsp13 to 0.5 μ M by SEC buffer.
2. Add the following reagents in turn to prepare the reaction mixture: ddH₂O (36.5 μ L), 5 \times ATPase reaction buffer (10 μ L), ATP (1 mM, 2.0 μ L), and [γ -³²P]ATP (~1 nM, 1 μ L) [18].
3. Add diluted nsp13 protein (0.5 μ M, 2 μ L) to the reaction mixture, incubate at 30 °C and start timing.
4. At each indicated time point, add 2 μ L quenching buffer (0.5 M EDTA) to the mixture to stop the reaction and place the mixture on ice.

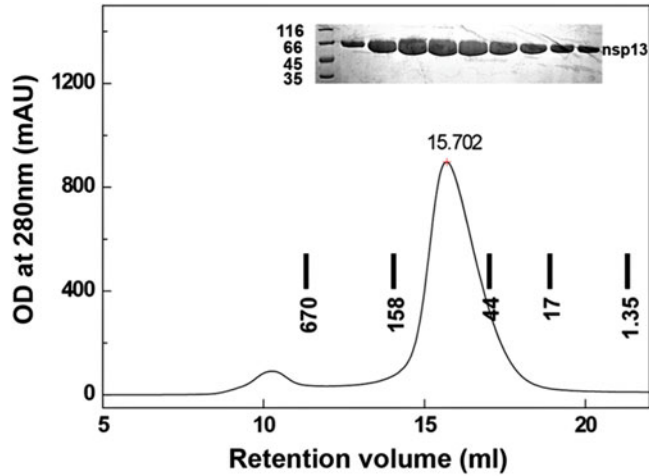


Fig. 2 Large-scale purification of MERS-CoV nsp13. MERS-CoV nsp13 eluted from Superdex 200 300/10 GL column precalibrated with gel filtration standards (thyroglobulin 670 kDa, γ -globulin 158 kDa, ovalbumin 44 kDa and myoglobin 17 kDa and vitamin 1.35 kDa (1350 Da)). Upper insert, SDS-PAGE analysis of the purified protein

5. Spot 1 μ L sample from the mixture on the thin-layer chromatography cellulose TLC plates and resolve with running buffer for 20 min.
6. Dry the plates and press the plate onto phosphor screen for 2 h. Analyze the result by storage phosphor screen and Typhoon Trio Variable Mode Imager (Fig. 3).

3.7 Helicase Assay of MERS-CoV nsp13

1. Dilute the purified MERS-CoV nsp13 to 1 μ M by SEC buffer (*see Note 4*).
2. Add the following reagents in turn: 10 \times helicase reaction buffer (1 μ L), H₂O (4 μ L), Trap RNA (3 μ M, 1 μ L), partial duplex RNA substrate (0.5 μ M, 1 μ L), and diluted nsp13 protein (0.5 μ M, 2 μ L), ATP (10 mM, 1 μ L). The final volume of each reaction mixture is 10 μ L [19].
3. Incubate the mixtures at 30 $^{\circ}$ C for 30 min.
4. Add 2.5 μ L 5 \times loading buffer to the mixture to stop the reaction.
5. Take 4 μ L sample from each reaction mixtures and load the samples onto 10% native PAGE gel.
6. Run the native PAGE gel at 100 V for 40 min on ice.
7. Scan the gel (Fig. 4).

3.8 Crystallization of MERS-CoV nsp13

Crystals of the unliganded MERS-CoV nsp13 diffracted the X-rays poorly, >3.6 \AA . The addition of 5'-triphosphate-15 dT DNA (ppp-15T) greatly improves the resolution.

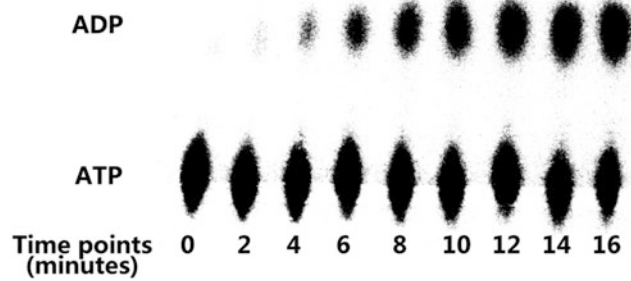


Fig. 3 ATPase activity of MERS-CoV nsp13. ATPase activity was measured by incubation at 30 °C for 0–16 min

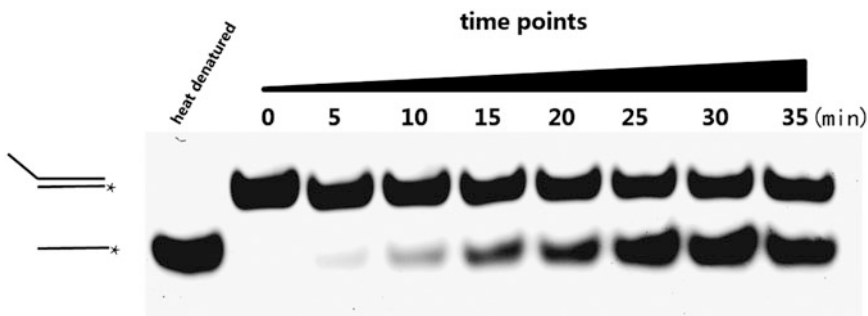


Fig. 4 Helicase activity of MERS-CoV nsp13. Activity was determined with the indicated RNA substrate (the asterisk marks the position of the HEX label). Samples were incubated at 30 °C for 0–35 min

1. Mix the purified MERS-CoV nsp13 with 5'-triphosphate-15T DNA (ppp-15T) with 1:1.5 molar ratio and incubate at 4 °C overnight.
2. Mix 1 μ L sample with 1 μ L reservoir buffer from the crystallization conditions screen kits, and incubate at 18 °C using the hanging-drop vapor-diffusion system.
3. Crystallize MERS-CoV nsp13 by mixing with the equal volume of reservoir buffer containing 0.1 M Tris-HCl (pH 8.5), 1 M $(\text{NH}_4)_2\text{SO}_4$, and 15% glycerol. Crystals grow to their maximum in a week (Fig. 5).

3.9 Determination of MERS-CoV nsp13 Crystal Structure

1. Highly redundant multi-wavelength anomalous diffraction data should be collected using the X-ray with wavelengths close to the absorption edge of zinc. High energy remote wavelength should be 1.2810 Å, peak wavelength: 1.2827 Å (two datasets were collected to improve the redundancy), and inflection wavelength 1.2831 Å.
2. Data processing and reducing by XDS Package and Truncate software from CCP4. The crystals belong to the space group $P6_122$, and contained two copies of nsp13 per asymmetric unit.

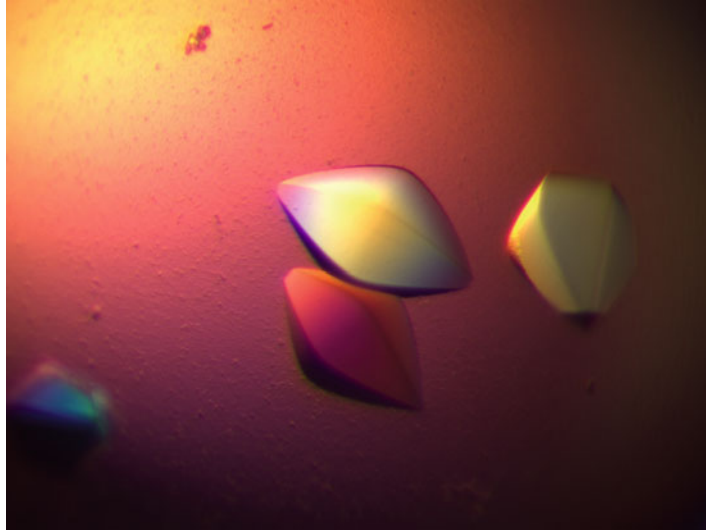


Fig. 5 Crystals of MERS-CoV nsp13. Nsp13 crystallized by incubation with 5'-triphosphate-15T DNA at 18°C using the hanging-drop vapor-diffusion system. The crystals are grown to the biggest in a week

3. An interpretable electron density map should be calculated using SHARP/autoSHARP [20].
4. Manually build the initial model of MERS-CoV nsp13 by Coot [21].
5. Collect native data with highest resolution (3.0 Å) using the X-rays with the wavelength of 0.978 Å.
6. Higher resolution structure should be solved by molecular replacement using the initial nsp13 structure as the searching model.
7. Manual model building with the improved electron density map. While most part of nsp13 can be located, the electron density of 1B subdomain is very weak, reflecting that this part is highly flexible.
8. Structure refinement to resolution limit of 3.0 Å using software PHENIX [22].

In the final model (Fig. 6), 145-230aa (the entire 1B domain) of molecule A are disordered, probably due to mobility of 1B and the lack of crystal contacts, whereas in molecule B, 591 out of 598 amino acids were located in the electron density maps (Fig. 7). Data collection and refinement statistics are summarized in Table 1.

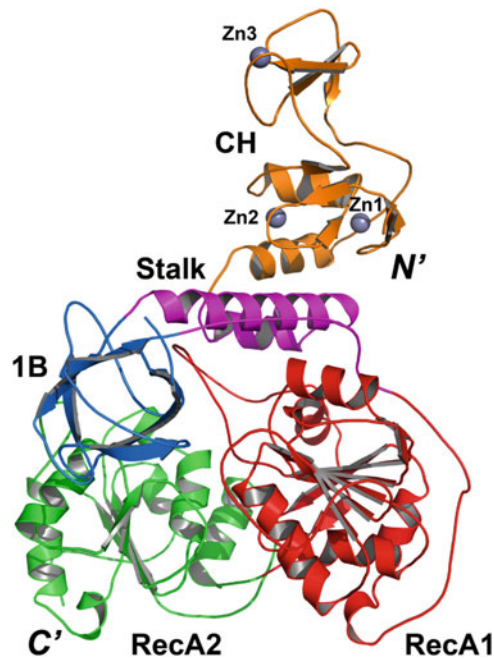


Fig. 6 Final model of MERS-CoV nsp13 structure. Model of MERS-CoV nsp13 containing CH (orange), Stalk (magenta), 1B (blue), RecA1 (red), and RecA2 (green) domains

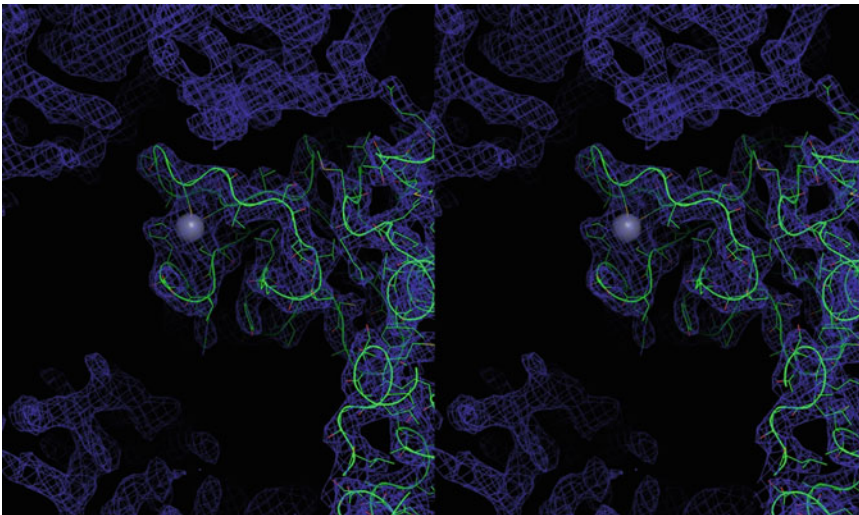


Fig. 7 Portion of the electron density map of MERS nsp13 crystal structure. A wall-eye stereo image of a portion of electron density map (zn3 binding site). 2Fo-Fc map is shown with blue mesh. The final model of MERS nsp13 (green) is superimposed. The zinc is shown with a gray sphere

Table 1
Data collection and refinement statistics

	MERS-CoV nsp13	MERS-CoV nsp13 (PDB ID: 5WWP)
Data collection	MAD phasing	Native data
Space group	P6 ₁ 22	P6 ₁ 22
<i>Cell dimensions</i>		
a, b, c (Å)	186.19, 186.19, 185.44	185.68, 185.68, 185.09
α, β, γ (°)	90.00, 90.00, 120.00	90.00, 90.00, 120.00
X-ray source	SLS X06DA	SSRF BL19UI
Wavelength (Å)	1.2827 (peak)	1.2810 (Hrem)
Data range (Å)	49.06–3.12	49.05–3.12
Reflections unique	63,859	63,771
R_{sym}^a (last shell)	0.29 (1.89)	0.30 (1.98)
CC(1/2)	99.7 (61.6)	99.3 (47.7)
$I/\sigma I$	13.80 (1.76)	10.66 (1.31)
Completeness (%) (last shell)	99.9 (99.7)	99.9 (99.7)
Redundancy (last shell)	21.15 (19.79)	12.91 (12.08)
Refinement		
Resolution range (Å)		48.95–3.00
% reflections in cross-validation		4.81
$R_{\text{work}}^b / R_{\text{free}}^c$ (last shell)		0.23, 0.28 (0.38, 0.41)

Atoms	
All atoms	8571
Protein	8540
Zinc	6
Solvent	25
<i>B</i> -factors average (Å ²)	68.1
Protein (Å ²)	68.1
Ligands (Å ²)	68.5
Solvent (Å ²)	90.2
r.m.s.d	
Bond lengths (Å)	0.015
Bond angles (°)	0.950
Validation	
MolProbity score	2.75, 88th percentile ^d
Clashscore, all atoms	15.39, 97th percentile ^d
% residues in favored regions, allowed regions, outliers in Ramachandran plot	91.8, 7.5, 0.7

^a $R_{\text{sym}} = \frac{\sum_{\text{hkl}} \sum_j |I_{\text{hkl},j} - I_{\text{hkl}}|}{\sum_{\text{hkl}} \sum_j I_{\text{hkl},j}}$ where I_{hkl} is the average of symmetry-related observations of a unique reflection

^b $R_{\text{work}} = \frac{\sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl}) - |F_{\text{calc}}(\text{hkl})||}{\sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl})|}$

^c R_{free} = the cross-validation *R* factor for 5% of reflections against which the model was not refined

^d100th percentile is the best among structures of comparable resolution; 0th percentile is the worst. For clash score the comparative set of structures was selected in 2004, for MolProbity score in 2006

4 Notes

1. When we prepare P1 virus in six-well plates, the medium in the wells always evaporated. Sealing the gap of the plate by medical tape can reduce the evaporation of medium (don't seal the gap completely, leave a small gap to keep the ventilation). Having a water trough in incubator also can reduce the evaporation of the medium.
2. The culture of insect cells sometimes was harassed by the contamination of bacteria or other microbes. To avoid the contamination, we treat the conical flasks not only by conventional autoclave sterilization, but also leave the 3 L conical flask (sealed by tinfoil) in the oven at 200 °C for 3–5 h before using.
3. To remove nucleic acids bound to nsp13, we used the lysis buffer containing high concentrate salt; this is a key step and improves the crystallization of nsp13 [10]. In practice, when sonicated in the buffer containing high concentrate salt, we found that the SUMO-tagged recombinant proteins lead the supernatant of the high-5 cell lysate to be turbid, which finally blocks the affinity columns. We have tried four concentrations of NaCl in lysis buffer, including 300 mM, 500 mM, 1 M, and 1.5 M. The first three concentrations of NaCl render the supernatant to be unable to use, we can't improve it by high-speed centrifugation ($47,850 \times g$), and it also can't be filtered by 0.45 μm syringe filter. The last concentration, 1.5 M NaCl in lysis buffer, could generate a bit better supernatant of cell lysates than other three concentrations of salt. We centrifuge the supernatant twice, then can filter it by 0.45 μm syringe filters (100 mL supernatant consumed about 8–10 filters). This clarified supernatant can flow through the affinity columns well.
4. The results of helicase assay always face the contamination of background fluorescence. Keep the gel from contacting any items containing fluorescence in the lab, including fluorescent dyes, some plastic boxes, hand towel, and so on.

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