Structural and Biochemical Characterization of Endoribonuclease Nsp15 Encoded by Middle East Respiratory Syndrome Coronavirus

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ABSTRACT Nonstructural protein 15 (Nsp15) encoded by coronavirus (CoV) is a nidoviral uridylyate-specific endoribonuclease (NendoU) that plays an essential role in the life cycle of the virus. Structural information on this crucial protein from the Middle East respiratory syndrome CoV (MERS-CoV), which is lethally pathogenic and has caused severe respiratory diseases worldwide, is lacking. Here, we determined the crystal structure of MERS-CoV Nsp15 at a 2.7-Å resolution and performed the relevant biochemical assays to study how NendoU activity is regulated. Although the overall structure is conserved, MERS-CoV Nsp15 shows unique and novel features compared to its homologs. Serine substitution of residue F285, which harbors an aromatic side chain that disturbs RNA binding compared with that of other homologs, increases catalytic activity. Mutations of residues residing on the oligomerization interfaces that distort hexamerization, namely, N38A, Y58A, and N157A, decrease thermostability, decrease affinity of binding with RNA, and reduce the NendoU activity of Nsp15. In contrast, mutant D39A exhibits increased activity and a higher substrate binding capacity. Importantly, Nsp8 was found to interact with both monomeric and hexameric Nsp15. The Nsp7/Nsp8 complex displays a higher binding affinity for Nsp15. Furthermore, Nsp8 and the Nsp7/Nsp8 complex also enhance the NendoU activity of hexameric Nsp15 *in vitro*. Taking the findings together, this work first provides evidence on how the activity of Nsp15 may be functionally mediated by catalytic residues, oligomeric assembly, RNA binding efficiency, or the possible association with other nonstructural proteins.

IMPORTANCE The lethally pathogenic Middle East respiratory syndrome coronavirus (MERS-CoV) and the severe acute respiratory syndrome coronavirus (SARS-CoV) pose serious threats to humans. Endoribonuclease Nsp15 encoded by coronavirus plays an important role in viral infection and pathogenesis. This study determines the structure of MERS-CoV Nsp15 and demonstrates how the catalytic activity of this protein is potentially mediated, thereby providing structural and functional evidence for developing antiviral drugs. We also hypothesize that the primase-like protein Nsp8 and the Nsp7/Nsp8 complex may interact with Nsp15 and affect enzymatic activity. This contributes to the understanding of the association of Nsp15 with the viral replication and transcription machinery.

KEYWORDS MERS-CoV, endoribonuclease, crystal structure, oligomerization

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A decade after the severe acute respiratory syndrome coronavirus (SARS-CoV) epidemic, a zoonotic coronavirus called Middle East respiratory syndrome coronavirus (MERS-CoV) circulated throughout the human population (1, 2). The lack of CoV-specific antiviral drugs or an effective vaccine has severely hampered efforts to combat the spread of this virus. It is therefore important to study the life cycle of the virus and the roles that viral proteins play in its propagation so that they can be targeted for the development of antiviral therapeutics.

CoVs are enveloped, single-stranded, positive-sense RNA viruses (3). The genomes of CoVs are the largest among RNA viruses and range between 26 and 32 kb (4). Almost two-thirds of the genome encompasses two large open reading frames (ORFs), ORF 1a and ORF 1b, which encode 16 nonstructural proteins (Nsps) that play essential roles in coronavirus RNA replication and transcription. A unique feature shared by all CoVs is that the ribosome undergoes a -1 frameshift following the translation of Nsp10. This results in the production of a large polypeptide, 1ab, that is then proteolytically processed to produce the 16 viral Nsps (5, 6). In addition to these Nsps, several structural and accessory proteins are synthesized from ORFs located at the 3' end of the viral genome. These ORFs are transcribed into a nested set of subgenomic RNAs that are ultimately translated into structural proteins (7, 8).

Nonstructural protein 15 (Nsp15) is a nidoviral uridylyte-specific endoribonuclease (NendoU) (9). It was reported to preferentially cleave 3' of uridylylates over cytidylates and generate a 2',3'-cyclic phosphate and 5'-OH ends (10). The activities of SARS-CoV Nsp15 and mouse hepatitis virus (MHV) Nsp15 were reported to be significantly stimulated by Mn^{2+} (11, 12). Previous studies have demonstrated that the recombinant SARS-CoV Nsp15 and MHV Nsp15 both existed in a monomer-trimer-hexamer equilibrium in solution, with the hexamer possessing endoribonuclease activity (10, 12, 13). Crystal structures of Nsp15 from SARS-CoV and MHV reveal that hexamerization of the protein lends structural support to maintain integrity of the active site. Two loops in the catalytic domain (residues 234 to 249 and 276 to 295 in SARS-CoV Nsp15) are packed against each other and are stabilized by intimate intermonomer interactions (10, 12, 13). N-terminal truncation of SARS-CoV Nsp15 resulted in an inactive monomeric state, with the catalytic loop containing two catalytic residues, H234 and H249, falling into the active-site cleft (14), which provides structural evidence to support that the hexamer is the active form.

Several *in vivo* studies have also been conducted to evaluate the function of Nsp15. Loss-of-function mutations in the catalytic sites of MHV Nsp15 reduced subgenomic RNA accumulation and profoundly attenuated virus infection, and similar results were also observed with SARS-CoV and *Arterivirus* (15–17). More recently, Nsp15 from SARS-CoV was found to be an inhibitor of mitochondrial antiviral signaling adaptor (MAVS) inducing apoptosis (18). In addition, Nsp15 from both MHV and SARS-CoV can interact with retinoblastoma tumor suppressor protein (pRb), thus affecting cell cycle-associated gene expression (19). It is therefore likely that Nsp15 impacts not only the viral life cycle but also the metabolic status and immune response of the host cells. Moreover, MHV Nsp15 has been demonstrated to colocalize and interact with the viral primase Nsp8 and polymerase Nsp12 *in vivo* (20), suggesting its possible involvement in RNA replication and transcription.

Here, we first describe the crystal structure of Nsp15 from MERS-CoV refined to a 2.7-Å resolution. Crucial residues within the active-site pocket and interprotomer interaction surfaces were found to play essential roles in the enzymatic function of the protein by directly regulating catalysis or imparting oligomeric arrangement and stability, thus modulating the substrate-RNA binding process. Furthermore, we demonstrated that primase-like Nsp8 and the Nsp7/Nsp8 complex could interact with MERS-CoV Nsp15 and affect NendoU activity, indicating a possible association of Nsp15 with other important nonstructural proteins that are involved in RNA replication and transcription.

TABLE 1 Data collection and refinement statistics

Parameter ^a	Value for MERS-Nsp15 ^b
Data collection statistics	
Unit cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	145.6, 145.6, 96.7
α , β , γ (°)	90.0, 90.0, 120.0
Space group	H3
Wavelength used (Å)	0.9798
Resolution (Å)	50.0–2.70 (2.75–2.70)
No. of reflections	
Total	222,454 (9,905)
Unique	21,016 (1,065)
Completeness (%)	100.0 (100.0)
Avg <i>I</i> / σ (<i>I</i>)	10.6 (2.33)
CC _{1/2}	0.952 (0.796)
<i>R</i> _{merge} (%)	10.4 (69.9)
Refinement statistics	
No. of reflections used [$\sigma(F) > 0$]	19,908
<i>R</i> _{work} (%)	17.5
<i>R</i> _{free} (%)	24.7
RMSD	
Bond distance (Å)	0.011
Bond angle (°)	1.453
Avg B value (Å ²)	44.62
No. of:	
Protein atoms	5,484
Ligand atoms	17
Solvent atoms	252
Ramachandran plot (%)	
Favored regions	93.66
Allowed regions	6.19
Outlier regions	0.15

^a $R_{\text{merge}} = \sum_h \sum_l |I_{hl} - \bar{I}_h| / \sum_h \sum_l I_{hl}$, where I_{hl} is the mean of observations I_{hl} of reflection h . $R_{\text{work}} = \sum [|F_o(\text{obs})| - |F_o(\text{calc})|] / \sum |F_o(\text{obs})|$. R_{free} is an *R* factor for a preselected subset (5%) of reflections that was excluded in the refinement.

^bNumbers in parentheses are corresponding values for the highest-resolution shell.

RESULTS

Overall structure of MERS-Nsp15. The recombinant full-length MERS-CoV Nsp15 was expressed, purified to homogeneity, crystallized in the H3 space group and diffracted to a 2.7-Å resolution. The final coordinates consist of protein residues 1 to 341 with good crystallographic quality (Table 1). For simplicity, MERS-Nsp15 and SARS-Nsp15 are used to represent MERS-CoV Nsp15 and SARS-CoV Nsp15, respectively, below.

The overall structure of MERS-Nsp15 consists of three distinct domains (Fig. 1A) and shares homology with the reported SARS-Nsp15 and MHV Nsp15 (10, 12, 13). Residues 1 to 60 are folded into a small N-terminal domain, in which three antiparallel β -strands (β 1 to β 3) are observed forming a curved β -sheet with two short α -helices (α 1 and α 2) right beneath it. The N-terminal domain is followed by a middle domain that contains a central β -sheet (β 4, β 7, β 8, and β 11) flanked by two small α -helices (α 3 and α 4) on either side. Two short β -strands (β 9 and β 10) arranged in a β -hairpin are located at the interface of the central domain and the C-terminal domain. Residues I190 to R341 within the C-terminal domain pack into two β -sheets consisting of β -strands β 13 to β 15 and β -strands β 16 to β 18, which constitute the catalytic-site cleft located at one side of the C-terminal domain. A group of five small α -helices (α 5 to α 9) packed at the other side of the domain face the concave surface of the β -sheets.

Crystal packing of MERS-Nsp15 is suggestive of a hexamer model. A dimer of trimers constitutes a hexameric architecture, with the crystallographic 3-fold axis passing through the center of the hexameric assembly (Fig. 1B and C). The N-terminal domains of the protomers within the two trimers pack back-to-back into a hexamer, placing the C-terminal domains that harbor the active site at the apexes of the cloverleaf-like

FIG 1 Overall structure of MERS-Nsp15. (A) Cartoon representation and schematic diagram of the overall structure of a protomer. A schematic diagram of the domain boundaries in the amino acid sequence is shown above the cartoon. The N-terminal domain, middle domain, and C-terminal domain are colored red, green, and blue, respectively. The surface transparency is set to 20%. (B) Side view of the cartoon representation of the hexamer. Three protomers within a trimer are colored green, yellow and blue, with the other trimer colored gray. (C) Side view and top view of the distribution of the N-terminal domain, middle domain, and C-terminal domain within the hexamer by surface representation. Six protomers are colored differently. The N-terminal domain, middle domain, and C-terminal domain within one protomer are colored red, green, and blue and are labeled N, M, and C, respectively. Six protomers form a hexamer through the N- to N-terminal interaction.

symmetry (Fig. 1C). Within the trimer assembly, the N-terminal domain of one protomer packs with a cleft between the central domain and the C-terminal domain of an adjacent protomer (Fig. 1C).

The structure of Nsp15 is conserved, with the root mean square deviations (RMSD) of C α atoms at 1.23 Å and 1.17 Å between monomeric MERS-Nsp15 and SARS-Nsp15 as well as MHV Nsp15. The RMSD for comparison of the trimeric MERS-Nsp15 with SARS-Nsp15 and MHV Nsp15 are 2.49 Å and 3.02 Å, respectively (Fig. 2A). Three domains within the monomer are also conserved, with the RMSD of C α atoms of aligned residues ranging from 0.71 to 1.25 Å (Fig. 2B). Additionally, many of the conserved residues that may contribute to the function of the protein (key residues that make up the catalytic site within the C-terminal domain and polar residues forming hydrogen bond networks) may be essential for protein function (Fig. 3).

Key residues within the catalytic site. To better uncover the structural information within the catalytic site, the structures of MERS-Nsp15 and SARS-Nsp15 were superimposed (Fig. 4A). Three highly conserved residues clustering in a positively charged groove are known to drive NendoU-mediated catalysis: the two catalytic histidines, H231 and H246, are located on a long, convoluted loop wedged between two adjacent β -sheets, and the third catalytic residue, K286, resides on strand β 15. The spatial arrangements reveal that residues S290 and Y339 in MERS-Nsp15 correspond to residues S293 and Y342 in SARS-Nsp15, which are postulated to interact with the substrate and confer uridylyate specificity (10), suggesting that there is conserved recognition for uridylyate. However, not all residues within the active site are conserved among coronaviruses. Several notable differences include the phenylalanine (F285) in MERS-Nsp15 that is located on strand β 15 within one of the 2 β -sheets at one end of the catalytic site. The aromatic side chain of F285 protrudes outward at a roughly perpendicular angle to the β -strand plane, compared to the corresponding serine (S288) in SARS-Nsp15. A threonine (T241), which is spatially located next to F285 and H246, occupies the position of Q244 in SARS-Nsp15, whereas an isoleucine is located in the corresponding position in MHV-Nsp15 (Fig. 3 and 4A). In addition, an arginine

FIG 2 Comparison of MERS-Nsp15 with SARS-Nsp15 and MHV Nsp15. (A) The structures of MERS-Nsp15, SARS-Nsp15 (PDB code 2H85), and MHV (PDB code 2GTH) Nsp15 are superimposed together. Monomers and trimers are shown separately, and RMSD of C α atoms are listed. (B) Three domains of the monomer for MERS-Nsp15, SARS-Nsp15, and MHV Nsp15 are aligned, and RMSD of C α atoms are listed.

(R341) in MERS-Nsp15 replaces a lysine (K344) in SARS-Nsp15 (Fig. 3). Thus, residues that are not conserved revealed unique features in MERS-Nsp15.

Functional characterization of MERS-Nsp15 and its active-site mutants. To explore the functional importance of residues within the catalytic site, the residues listed in Fig. 4A were replaced by alanine. Gel filtration revealed that all mutants shared elution profiles similar to that of the wild-type Nsp15. The elution profiles exhibited a dominant peak corresponding to a hexamer (data not shown). To further investigate the thermostability of these mutants, we conducted a differential scanning fluorimetry (DSF) assay, which has been used to assess interactions among protein subunits (21). The denaturation profiles of wild-type Nsp15 and its mutants in Fig. 4A revealed that they all exhibited a major transition at the melting temperature (T_m) of 46°C, which is suggestive of a native fold (Fig. 4C). Taking the data together, the mutation of key residues in the active site had no effect on the oligomeric assembly and stability of MERS-Nsp15.

We subsequently investigated the NendoU activity of these active-site mutants using fluorescent resonance energy transfer (FRET) assays, in which a substrate containing the nucleotide rU was used. Alanine substitution of the three highly conserved residues (H231, H246, and K286), as well as the residues located in the immediate surrounding regions (Y339, T241, and R341), decreased RNase activity to the background level (Fig. 4D and E). Notably, when T241 was replaced with the corresponding residue glutamine in SARS-Nsp15, T241Q exhibited wild-type activity. Mutating R341 to lysine, the corresponding residue in SARS-Nsp15, decreased NendoU activity to ap-

FIG 3 Sequence alignment of MERS-Nsp15 with Nsp15s of coronaviruses and arteriviruses. Key residues within the catalytic center are marked by red arrowheads, and residues in the subunit interfaces are marked by blue circles. Strictly conserved residues are depicted in white characters on a black background. Secondary-structure elements are shown above the alignment (helices are represented by squiggles, β -strands by arrows, and turns by the letters TT). Sequences are aligned using ClustalW (30), and the figure was drawn with ESPript (31). NCBI accession numbers are as follows: [YP_009047226](#) for Middle East respiratory syndrome-related coronavirus (MERS-CoV), [AGT21317](#) for SARS coronavirus (SARS-CoV), [NP_740619](#) for murine hepatitis virus strain A59 (MHV), [AGT21366](#) for human coronavirus 229E (HCoV-229E), [AIM47753](#) for porcine epidemic diarrhea virus (PEDV), [AGZ84515](#) for feline infectious peritonitis virus (FIPV), [ABG89333](#) for transmissible gastroenteritis virus virulent Purdue (TGEV), and [NP_705592](#) for equine arteritis virus (EAV).

FIG 4 Identification and characterization of residues within the catalytic center of MERS-Nsp15. (A) Structural superposition of MERS-Nsp15 and SARS-Nsp15 (PDB code 2H85) (13). The structure of MERS-Nsp15 is colored magenta, and the structure of SARS-Nsp15 is colored yellow. The catalytic center of MERS-Nsp15 superimposed with SARS-Nsp15 is enlarged in panel A (the cartoon transparency is set to 20%). Residues discussed in this paper are labeled with stick representations (magenta, MERS-Nsp15; yellow, SARS-CoV Nsp15). Equivalent residues located in the catalytic site of SARS-Nsp15 are listed at the bottom. (B) Protein-RNA binding profiles and the inhibitory effects of the cleavage of the fluorescent substrate by RNAs R1, R2, and R3. R1 contains 20 rU nucleotides and exhibits a K_d value of 818.28 ± 50.39 nM. R2 binds to Nsp15 with a K_d value of $1,190.52 \pm 137.91$ nM. R3 exhibits a K_d value of 932.49 ± 49.40 nM. The activity of the wild-type Nsp15 in the absence of R1, R2, or R3 is set to 100%. The fluorescence intensity was measured at each RNA (R1 to R3) concentration, and the values shown are the averages from three measurements. (C) DSF profile of wild-type Nsp15. All mutants listed in panel A share melting temperatures and DSF profiles similar to those of wild-type Nsp15. (D) NendoU activity profile for the mutants with alanine substitution of residues located within the catalytic site. (E) NendoU activity profile for active-site mutants with alanine substitutions and the corresponding residue in SARS-Nsp15.

proximately 50% of the wild-type protein activity (Fig. 4E). Interestingly, the catalytic rate of Nsp15 slightly increased to 1.34 ± 0.06 nM s^{-1} when F285 was replaced by alanine, which is found in the equivalent position in equine arteritis virus (EAV) (Fig. 4). We also replaced F285 with a serine, a residue that is present in all other coronaviruses at this position. Serine substitution resulted in an increase in catalytic activity to 1.63 ± 0.07 nM s^{-1} (Fig. 4E). Moreover, alanine substitution of S290, for which the corresponding residue was postulated to confer uridylylase specificity, reduced the activity to 67% of the wild-type Nsp15 activity (Fig. 4D).

How does the disruption of active-site residues affect NendoU activity? We next used a fluorescence polarization (FP) assay to assess the RNA binding ability of these mutants. Three different RNAs (R1 to R3) were designed to identify the one that binds most strongly to Nsp15: R1 contains 20 rU nucleotides, R2 is derived from the conserved transcriptional regulatory sequence (TRS) of the viral genome, and R3 is a double-stranded RNA annealed by R2 and its complementary strand. Among the three oligomers, Nsp15 exhibited the highest binding affinity to RNA R1 (Fig. 4B). RNA R1 also had the highest inhibitory effect on NendoU activity in the FRET-based assays, suggesting that it possesses the strongest binding ability, and it was thus used in all FP

TABLE 2 Catalytic activity and RNA binding properties of wild-type Nsp15 and its active-site mutants^a

MERS-Nsp15	Catalytic rate (nM/s)	K_d value (nM)	Hill coefficient (<i>n</i>)
Wild type	1.25 ± 0.06		
K286A	ND ^b	818.28 ± 50.39	2.24 ± 0.24
H246A	ND	916.47 ± 102.13	2.07 ± 0.53
Y339A	Very low	1,029.04 ± 102.73	2.16 ± 0.44
H231A	ND	811.01 ± 51.00	3.11 ± 0.64
S290A	0.83 ± 0.02	1,156.49 ± 94.12	2.38 ± 0.37
F285A	1.34 ± 0.06	678.63 ± 36.75	2.72 ± 0.43
F285S	1.63 ± 0.07	616.28 ± 33.09	2.39 ± 0.34
T241A	ND	997.11 ± 121.47	2.30 ± 0.64
T241Q	1.17 ± 0.04	798.96 ± 53.70	2.44 ± 0.47
R341A	ND	1,037.28 ± 72.79	2.57 ± 0.44
R341K	0.61 ± 0.06	807.61 ± 51.92	2.65 ± 0.51

^aAll Nsp15 mutants for the FP assays harbored the K286A mutation to avoid the possible digestion of the RNA substrate. The K_d value and Hill coefficient of the wild-type protein were determined and are represented by mutant K286A. The K_d and Hill coefficient values (means ± SD) were obtained by fitting the data to the Hill equation.

^bND, not detected.

assays to assess the RNA-Nsp15 interactions in this study (Fig. 4B). Mutants K286A, H246A, H231A, Y339A, T241A, and R341A, which exhibited no activity, were all able to bind RNA, indicating that their decreased activity was not caused by substrate binding but possibly was caused by the directly catalytic function of these residues (Table 2). Of note, the F285A and F285S mutants exhibited stronger binding than the wild-type protein, which may explain why the NendoU activity of these two mutants increased (Fig. 4E; Table 2). Consistently, mutant S290A, which exhibited partly diminished activity, conferred a moderate decrease in RNA binding ability. In conclusion, these observations corroborated that mutation of critical residues within the active site, through either directly disrupting catalysis or impacting RNA binding affinity, may influence NendoU activity.

Oligomeric assembly affects RNA binding and NendoU activity. To gain insight into the correlation between NendoU activity and the distinct oligomeric forms, we examined the interprotomer surfaces and disrupted several key residues that contribute to the hydrogen bond networks: (i) the $\delta 1$ oxygen atom of N38 on strand $\beta 3$ in the N-terminal domain bonds with the backbone nitrogen atom of G95 on the loop connecting strand $\beta 6$ with strand $\beta 7$, (ii) the $\delta 2$ oxygen atom of D39 on strand $\beta 3$ bonds with the $\gamma 1$ oxygen atom of T48 on the loop connecting strand $\beta 3$ with helix $\alpha 2$ in the middle domain of another protomer, (iii) the nitrogen atom of $\delta 2$ N157 on the turn connecting strand $\beta 10$ with strand $\beta 11$ stacks against the $\gamma 1$ oxygen atom of T278 on $\beta 14$, and (iv) the $\eta 1$ oxygen atom of Y58 on helix $\alpha 2$ stacks against the ϵ oxygen atom of E263 on strand $\beta 13$ in the C-terminal domain (Fig. 5). Within the interface of the two trimers, L2 and E3 on helix $\alpha 1$ as well as V26 on strand $\beta 1$ of protomer B pack face-to-face against E3 and L2 on helix $\alpha 1$ as well as N52 on helix $\alpha 2$ of its counterpart protomer A, respectively. Moreover, the N terminus of subunit C is close to monomer A, with the closest atom-to-atom distance being 3.2 Å between residues A112 in protomer C and N110 in protomer A, suggesting that this close proximity may assist in the hexamer assembly process (Fig. 5).

To validate the significance of the interfaces, analytical ultracentrifugation (AUC) analysis was conducted to assess the oligomeric forms of soluble and stable variants. Mutant D39A existed primarily as a hexamer, while mutant E263A was distributed equally between the monomer and hexamer states. By comparison, mutants N38A, Y58A, and N157A existed predominately as monomers (Fig. 6A). DSF assays revealed that the denaturation profiles of the hexameric proteins (wild type and D39A Nsp15) indicated a melting temperature (T_m) of 46°C. E263A denatured at 43°C, while the T_m shifted to approximately 37 to 38°C for the monomeric mutants N38A, N157A, and Y58A (Fig. 6B). Taking the data together, we inferred that the hexameric form rendered the protein more stable and may thus impact protein function.

FIG 5 Stick representation of residues involved in the interprotomer interaction of MERS-Nsp15. A side view of the surface representation of the interactions within three protomers is shown. Protomers A, B, and C are colored blue, yellow, and magenta, respectively. Residues involved in the subunits interaction are labeled with stick representations (blue, subunit A; yellow, subunit B; and magenta, subunit C [the cartoon transparency is set to 20%]). Four contact regions are boxed and enlarged. The atomic distances were measured with PyMOL.

In an effort to evaluate the functional state and demonstrate how oligomeric assembly affects NendoU activity, the RNA binding ability and the NendoU activity of these variants were further assessed. The results of the FP and FRET-based assays showed that the hexameric mutant D39A bound to RNA R1 with a binding affinity (626.22 ± 24.80 nM) higher than that of the wild-type protein, which may explain why mutant D39A exhibited greater enzymatic activity than the wild-type Nsp15. Mutant E263A, which displayed an equal distribution between the monomer and hexamer states, retained an impaired binding affinity ($1,013.43 \pm 77.38$ nM), matching its moderately inhibited activity. Consistently, associations between the monomeric variants (N38A, Y58A, and N157A) and RNA R1 were barely detectable, and the activities of the monomeric mutants were abolished (Fig. 6C and D). Altogether, the correlation between RNA binding affinity and NendoU activity showed that RNA binds only to

FIG 6 Biochemical characterization of mutants critical for the oligomeric assembly of MERS-Nsp15. (A) Analytical ultracentrifuge (AUC) analysis of mutants containing mutations located at interaction surfaces. AUC profiles of the wild-type protein and the D39A, E263A, N38A, Y58A, and N157A mutants are shown. The first peak at approximately 40 kDa represents the position of the monomer, and the second peak at approximately 240 kDa represents the position of the hexamer. (B) DSF profiles of wild-type Nsp15 and mutants D39A, E263A, N38A, Y58A, and N157A. (C) Protein-RNA binding profiles of the Nsp15 mutants determined by fluorescence polarization (FP). Alanine substitution was performed at K286 in the wild type and mutants (D39A, E263A, N38A, Y58A, and N157A) for this assay to prevent substrate digestion during the experiment. (D) NendoU activity profiles for the mutants related to oligomeric assembly.

TABLE 3 Catalytic activity and RNA binding properties of wild-type Nsp15 and its subunit interface mutants^a

MERS-Nsp15	Catalytic rate (nM/s)	K _d value (nM)	Hill coefficient (n)
Wild type	1.25 ± 0.06		
K286A	ND ^b	818.28 ± 50.39	2.24 ± 0.24
N38A	0.38 ± 0.04	ND	ND
D39A	1.37 ± 0.04	626.22 ± 24.80	2.15 ± 0.17
N157A	ND	ND	ND
Y58A	0.25 ± 0.03	ND	ND
E263A	0.89 ± 0.06	1,013.43 ± 77.38	2.09 ± 0.20

^aAll Nsp15 mutants for the FP assays harbored the K286A mutation to avoid digestion of the RNA substrate. The K_d value and Hill coefficient of the wild-type protein were measured and are represented by mutant K286A. The K_d and Hill coefficient values (means ± SD) were obtained by fitting the data to the Hill equation.

^bND, not detected.

proteins in the hexameric state, indicating that oligomeric assembly may regulate NendoU activity by impacting its RNA binding ability (Table 3).

Effects of Mn²⁺ and Mg²⁺ on MERS-Nsp15. We further investigated the effect of Mn²⁺ on the activity of MERS-Nsp15. A significant increase in activity was observed as the concentration of Mn²⁺ was increased from 0 to 15 mM, with a 3-fold increase in activity under 10 mM Mn²⁺ (Fig. 7A). Fluorescence polarization (FP) assays were then conducted to examine the Nsp15-RNA binding affinity with increasing Mn²⁺ concentration. The Nsp15-RNA binding affinity increased with the addition of Mn²⁺, revealing that the function of Mn²⁺ may be a result of the increasing substrate binding ability of Nsp15 (Fig. 7B).

To determine whether Mg²⁺ can also affect the activity of MERS-Nsp15, we performed FRET-based assays and FP assays in which Mg²⁺ concentrations were increased. The catalytic activity of Nsp15 increased slightly in the presence of 15 mM Mg²⁺ (Fig. 7C). Consistently, the Nsp15-RNA binding ability was similar in the presence or absence of Mg²⁺ (Fig. 7D), indicating that the effect of Mg²⁺ *in vitro* was minor compared to the effect of Mn²⁺.

FIG 7 Effects of Mn²⁺ and Mg²⁺ on MERS-Nsp15. (A) Catalytic rate profile for the effects of Mn²⁺ on the activity of MERS-Nsp15. (B) Nsp15 (K286A)-RNA binding profiles for Nsp15 with increasing Mn²⁺ concentration determined via fluorescence polarization. (C) Catalytic rate profile for the effects of Mg²⁺ on the activity of MERS-Nsp15. (D) Nsp15 (K286A)-RNA binding profiles for Nsp15 with increasing Mg²⁺ concentrations determined via fluorescence polarization.

FIG 8 Influence of MERS-CoV Nsp8 and the Nsp7/Nsp8 complex on MERS-Nsp15. (A) Pull-down assays detecting the interaction of the Nsp12/Nsp15, Nsp8/Nsp15, or Nsp8/Nsp15 mutants. (B) MST binding curves for the titration of fluorescently labeled Nsp15 into Nsp8 (green) and Nsp12 (orange). Error bars showing SD were calculated from triplicate experiments. (C) Determination of the binding affinities of Nsp15 with Nsp7 (black) and the Nsp7/Nsp8 complex (blue) via MST assays. (D) Effects of Nsp8 and the Nsp7/Nsp8 complex on the endoribonuclease activity of MERS-Nsp15. (E) Effects of Nsp8 and the Nsp7/Nsp8 complex on the catalytic rate of Nsp15 mutants.

Effects of CoV replicase-transcriptase complex-associated protein Nsp8 and the Nsp7/Nsp8 complex on Nsp15. In agreement with previous evidence that MHV Nsp15 strongly colocalizes with primase Nsp8 as well as polymerase Nsp12, immunoprecipitation (IP) experiments also confirmed the binding of MHV Nsp15 to MHV Nsp12 as well as to MHV Nsp8 (20). Pull-down assays were further performed, and the direct interaction between Nsp15 and Nsp8 was monitored, but that between Nsp15 and Nsp12 was not monitored (Fig. 8A). To ascertain whether the interaction between Nsp15 and Nsp8 is dependent on the hexameric state of Nsp15, His-tagged Nsp8 was copurified with the Flag-tagged Nsp15 mutants (N38A, D39A, E263A, and N157A) harboring distinct oligomeric states and detected by pull-down assays. Nsp8 was shown to interact with each of the Nsp15 variants, indicating that both monomeric Nsp15 and hexameric Nsp15 interact with Nsp8 (Fig. 8A).

Nsp8 was cocrystallized with Nsp7 as a hexadecameric complex that displayed primase-like RdRp activity and dramatically increased the polymerase activity of Nsp12 (22, 23). We therefore examined whether Nsp15 binds to Nsp8 in the context of an Nsp7/Nsp8 complex and affects the endoribonuclease activity of Nsp15. To obtain the Nsp7/Nsp8 complex, purified Nsp7 and Nsp8 were incubated in a 1:1 molar ratio, and the elution peak representing the Nsp7/Nsp8 complex was isolated via gel filtration and corroborated by SDS-PAGE. We next detected and quantified the interaction of Nsp15 with Nsp8 and the Nsp7/Nsp8 complex by microscale thermophoresis (MST) assay, a sensitive method that can be used to monitor and quantify binding affinities of complex formation between proteins (24). The MST results revealed binding between Nsp15 and Nsp8 with a dissociation constant (K_d) value of $16.33 \pm 3.21 \mu\text{M}$ (Fig. 8B).

Notably, relatively enhanced affinity ($6.48 \pm 1.47 \mu\text{M}$) was observed for the Nsp7/Nsp8 complex titrated with fluorescently labeled Nsp15 (Fig. 8C). As a control, Nsp7 alone displayed no binding to Nsp15 by MST (Fig. 8C). However, we have been unable to detect binding between Nsp12 and the fluorescently labeled Nsp15 by the MST assay (Fig. 8B).

To test whether Nsp8 or Nsp7/Nsp8 impact the NendoU activity of Nsp15, we preincubated Nsp15 with Nsp8 and the Nsp7/Nsp8 complex at a 1:1 molar ratio before performing the FRET-based assays. The catalytic rate increased from $1.25 \pm 0.06 \text{ nM s}^{-1}$ to $1.88 \pm 0.09 \text{ nM s}^{-1}$ in the presence of Nsp8, while the Nsp7/Nsp8 complex increased the NendoU activity to $1.89 \pm 0.04 \text{ nM s}^{-1}$ (Fig. 8D). To further determine whether this enzymatic enhancement is related to the oligomeric state of Nsp15, we measured the catalytic rates of the variants D39A, E263A, and N157A in the presence of Nsp8 or the Nsp7/Nsp8 complex. Nsp8 enhanced the catalytic rate of the hexameric D39A protein by 58%, while the Nsp7/Nsp8 complex increased the catalytic rate by 54% (Fig. 8E). For E263A, the activity increased by 33% in the presence of Nsp8 and increased by 40% in the presence of Nsp7/Nsp8 (Fig. 8E). However, the activity of the monomeric N157A protein was similar in the presence of Nsp8 or Nsp7/Nsp8, demonstrating that this enhancement effect is highly dependent on the hexameric state of Nsp15.

DISCUSSION

In this study, we determined the crystal structure of MERS-Nsp15 refined to a 2.7-Å resolution. Structural examination of the active site and subunit interaction surfaces, together with biochemical characterization of the critical mutants, revealed that NendoU activity was mediated by (i) catalytic residues within the active-site pocket that were indispensable for NendoU activity, (ii) residues directly interacting with RNA, and (iii) residues providing structural support for hexameric assembly, which is the functionally active state that is responsible for its RNA binding ability.

Notably, several different features in MERS-Nsp15 compared to its homologs were identified. For example, the endoribonuclease activity of the variant F285A was slightly increased, and the activity of F285S increased about 31%, compared to that of the wild-type protein. Examination of the neighboring structure shows that replacement of F285 with alanine or serine likely offsets the steric obstruction of the aromatic side chain, which may spatially interfere with substrate binding in the pocket and thus likely widens the active-site cavity and facilitates access to the substrate. FP assays confirmed that F285A and F285S displayed increased binding affinity for RNA. In particular, the effect of serine substitution on activity enhancement was more dramatic than that of alanine substitution, possibly due to the fact that serine is hydrophilic, whereas alanine is hydrophobic. More importantly, sequence alignment demonstrated that at the equivalent position of F285, a serine is conserved among other coronaviruses as well as most arteriviruses, and an alanine is present at the equivalent position in equine arteritis virus (EAV) (Fig. 4). Thus, considering that MERS-Nsp15 is the only exception, it is plausible that F285 may exist in MERS-Nsp15 to mediate the function of this protein.

Another example is the hexameric mutant D39A, which exhibited higher activity than the wild-type protein. The corresponding mutant D39A from SARS-Nsp15 existed primarily as a monomer and displayed no activity (10). It is likely that apart from interacting with T48, D39 from SARS-Nsp15 also forms an ionic bond with R90, which contributes more to the interprotomer interactions, and this may explain why mutant D39A exists in different oligomeric states in SARS-Nsp15 and MERS-Nsp15. Remarkably, mutant D39A from MERS-Nsp15 exhibited a higher RNA binding affinity than wild-type protein, which may explain its higher activity.

Previous studies have demonstrated that the activity of Nsp15 from both SARS-CoV and MHV can be stimulated by Mn^{2+} (10–13). The activity of MERS-Nsp15 increased with the addition of Mn^{2+} , with enhanced RNA binding affinity (Fig. 7A and B). However, Mg^{2+} only slightly enhanced NendoU activity and did not influence RNA binding (Fig. 7C and D). It is plausible that NendoU activity could be regulated by RNA binding via Mn^{2+} . In contrast, structures of MERS-Nsp15 and its orthologs all show no

obvious metal binding sites, arguing against the direct involvement of Mn^{2+} . We speculate that Mn^{2+} may serve as a cofactor to stabilize the RNA-Nsp15 structure and thus enhance NendoU activity. Moreover, given that the intracellular concentration of these divalent metal ions is estimated to be within the micromolar range, these biochemical observations from *in vitro* studies may not be applicable to the *in vivo* environment. More information is needed to elucidate the roles that Mn^{2+} and Mg^{2+} play in the catalytic process and the possible mechanism.

If the hexamer assembles in such a way that three monomers first constitute a trimer and two trimers pack back-to-back into a hexamer as proposed, then the trimer state should be possible. Nonetheless, the trimer could not be isolated and biochemically characterized in our work or in previous studies, as the mutation of any of these residues (L2, E3, V26, N52, and N110), which are located at the preconceived trimer-trimer interfaces, yielded only proteins that were predominantly monomeric in solution (10). One possible explanation of this discrepancy may be that the trimer is highly unstable and is transient in nature.

Nsp15 interacted with Nsp8 during both the pulldown and MST assays. Remarkably, Nsp15 binds to the Nsp7/Nsp8 complex with a stronger affinity than to Nsp8. In contrast to previous IP results (20), both the pulldown and MST assays could not detect an interaction between Nsp15 and Nsp12, possibly due to extremely weak interaction. Moreover, given that Nsp12 can complex with Nsp8 and Nsp7 (20), another possible explanation might be that the detected interaction between Nsp12 and Nsp15 by IP was a result of the indirect binding of Nsp12 with Nsp8 instead of the direct binding with Nsp15.

In addition, NendoU activity may be increased by both Nsp8 and the Nsp7/Nsp8 complex, with this increase being observed only for the hexameric state of Nsp15, further strengthening the hypothesis that the hexameric state is the functional form. However, the mechanism through which NendoU activity is enhanced by Nsp8 and the Nsp7/Nsp8 complex remains unclear. It is likely that the interaction between Nsp8 and Nsp15 may provide a possible explanation (i.e., the direct interaction might induce a conformational change in Nsp15). Indeed, the SARS-CoV Nsp7/Nsp8 complex may bind RNA and has been shown to confer RNA binding ability to SARS-CoV Nsp12 (22, 23). We speculate, therefore, that Nsp8 or the Nsp7/Nsp8 complex may increase NendoU activity by enhancing the RNA binding ability of Nsp15. Further studies are required to elucidate mechanisms for the involvement of Nsp15 in the Nsp7/Nsp8/Nsp15 complex and the role that Nsp15 may play in viral replication/transcription complex machinery and coronavirus pathogenesis.

In conclusion, we provide the first structure of MERS-Nsp15, and our structure-function studies demonstrate crucial features and provide important novel insights into how NendoU activity is possibly mediated. Moreover, given that inhibitors targeting the catalytic sites may potentially interfere with endoribonuclease within host cells, this work also provides new insights on how drugs designed to disrupt interprotomer interaction surfaces may be applied.

MATERIALS AND METHODS

Construct design and molecular cloning. The sequence encoding the MERS-Nsp15 protein was optimized for expression in *Escherichia coli* and was synthesized by GenScript. A hexahistidine tag was inserted at the N-terminal end via PCR with the forward primer 5'-CATGCCATGGGCCACCACCACCA CCACGGCCTGGAAACATTGCGTTTAATG-3' and the reverse primer 5'-CCGCTCGAGTTATTGCAGGCGCGG ATAGAAGGTTTGAC-3'. The PCR product was then cloned into the pRSF-Duet1 vector between the NcoI and XhoI sites. Point mutations were introduced into the wild-type Nsp15 plasmid using the Fast Mutagenesis System (Transgene Biotech). Nsp15 and Nsp15 mutants (N38A, D39A, N157A, and E263A) possessing a Flag tag at the N terminus were constructed in a similar procedure.

Expression and purification of MERS-Nsp15. The sequence-verified MERS-Nsp15 plasmid was transformed into *E. coli* strain BL21(DE3) cells. The cells were cultured in LB medium containing kanamycin (100 mg liter^{-1}) until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.8. The cell culture was then cooled to 16°C and induced with 0.4 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). After 14 to 16 h of induction, the cells were harvested and lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 70 mM imidazole. The lysate was centrifuged at $25,000 \times g$ for 40 min, and the soluble supernatant was purified by immobilized metal ion affinity chromatography. The eluate was fractionated

via a Superdex 200 10/300 (GE Healthcare) column with buffer containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl. The protein was concentrated to 8 mg/ml for crystal screening or stored at -80°C for further use. The MERS-Nsp15 mutants described in this paper were expressed and purified following a protocol similar to that described above.

Recombinant MERS-CoV Nsp8 containing both glutathione S-transferase (GST) and hexahistidine tags and MERS-CoV Nsp7 with a GST tag were expressed and purified as previously reported for SARS-CoV Nsp7 and Nsp8 (23). To obtain the Nsp7/Nsp8 complex, purified Nsp7 and Nsp8 were incubated in a 1:1 molar ratio at 4°C for 1 h, and then the proteins were separated with a Superdex 200 10/300 column. The elution peak representing the Nsp7/Nsp8 complex was further corroborated by SDS-PAGE, and the oligomeric state was evaluated by analytical ultracentrifugation (AUC) analysis. MERS-CoV Nsp12 carrying both the N-terminal GST tag and the C-terminal hexahistidine tag was expressed in the baculovirus expression system (Bac-to-Bac system; Invitrogen) with the Sf9 insect cell line. The GST-fused Nsp12 was primarily purified via a glutathione affinity column and was then digested by thrombin protease overnight to release the GST tag. Nsp12 was then further purified using a Hi-Trap Q column (GE Healthcare) and a Superdex 200 10/300 column (GE Healthcare) to over 95% purity.

Crystallization and structure determination. MERS-Nsp15 crystals were grown at 289 K by the hanging-drop vapor diffusion method. Crystals were grown overnight in a mixture of 1 μl protein and 1 μl reservoir solution (4% [vol/vol] tacsimate [pH 4.0] and 12% [wt/vol] polyethylene glycol 3350). Crystals were transferred to a cryoprotected buffer (reservoir solution and 25% glycerol) and flash frozen in liquid nitrogen.

The data set for Nsp15 was collected at beamline BL19 in the Shanghai Synchrotron Radiation Facility (SSRF), and the data were processed and scaled using the XDS program suite (25). The native Nsp15 structure was solved by molecular replacement with the program Phaser of the CCP4 using MHV Nsp15 (PDB code 2GTH) as the search model, and the structure refinement was carried out with PHENIX (12, 26–28). Final refinement statistics are summarized in Table 1. Structural figures were drawn with PyMOL (29).

AUC analysis. Purified MERS-Nsp15 and mutant proteins were subjected to analytical ultracentrifugation (AUC) analysis in a buffer containing 20 mM Tris (pH 7.5) and 50 mM NaCl. Sedimentation rate experiments were conducted at 4°C in a proteomeLab XL-1 protein characterization system (Beckman Coulter). The data were collected at 45,000 rpm using an An-60 Ti rotor (Beckman Coulter) and processed according to a c(s) distribution model.

DSF assay. A differential scanning fluorimetry (DSF) assay was carried out in a real-time PCR machine (LightCycler480II). Each 20- μl reaction mixture contained 0.5 mg/ml purified protein. SYPRO orange was diluted to a $5\times$ final concentration. The temperature was held for 10 s and increased from 25°C to 95°C at a rate of $1^{\circ}\text{C}/\text{min}$. The average fluorescence intensity and melting temperature (T_m) from three measurements were determined and used.

RNA oligonucleotides synthesis. RNA oligonucleotides (R1 to R3) were synthesized and purified by Genewiz to over 95% purity. RNA R1 contains 20 rU nucleotides (5'-rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrU-3'). RNA R2 (5'-rUrUrArArCrGrArArCrU-3') is derived from the conserved sequence of the transcriptional regulatory sequence (TRS) of the MERS-CoV genome, and RNA R3 is a double-stranded RNA annealed by R2 and its complementary strand (5'-rArGrUrUrCrGrUrUrArA-3').

FP assay. RNA oligonucleotides used for the fluorescence polarization (FP) assays were labeled at the 3' end with Cy3 and diluted to a final concentration of 100 nM. Purified Nsp15 was diluted with buffer containing 20 mM Tris (pH 7.5) and 50 mM NaCl. The effects of divalent ions were detected in the presence of 0 mM, 2 mM, 5 mM, 10 mM, or 15 mM MnCl_2 or MgCl_2 . Reaction mixtures with a total volume of 100 μl were prepared, and FP assays were performed using a Perkin-Elmer Envision instrument with an excitation wavelength of 555 nm and an emission wavelength of 595 nm. All Nsp15 proteins used for the FP assays in this study harbored the K286A mutation, which abolishes NendoU activity, to avoid substrate digestion. The anisotropy values reported were the average of three replicates, and the means $\pm$ standard deviations (SD) are shown. Data were further analyzed in Origin 8.0 (OriginLab) using the Hill equation. The calculated dissociation constants ($K_{d,s}$) and the Hill coefficients are listed in Table 2.

Endoribonuclease assay. Real-time endoribonuclease assays were performed using fluorescent resonance energy transfer (FRET) as described previously (12). The substrate (5'-6-FAM-dArUdAdA-6-TA MRA-3') was purchased from TaKaRa. The substrate had a carboxyfluorescein (FAM) at the 5' end and a tetramethylrhodamine (TAMRA) at the 3' end, which quenches the FAM fluorescent emission at 518 nm. The cleavage of the substrate leads to increasing fluorescence emission at 518 nm. The cleavage reaction was performed at room temperature, and the mixture contained 0.2 μM protein and 1.2 μM RNA substrate in a final volume of 100 μl . The fluorescence intensity over time was monitored with the EnSpire Multimode plate reader system (PerkinElmer) with an excitation wavelength of 498 nm and an emission wavelength of 518 nm.

The endoribonuclease assays of wild-type Nsp15 and its mutants were performed in buffer containing 50 mM Tris (pH 7.5) and 50 mM KCl. Nsp8, the Nsp7/Nsp8 complex, Nsp12, and the S protein (spike protein of MERS-CoV) were adjusted to a 1:1 molar ratio with Nsp15 at a final concentration of 0.2 μM . Nsp15 was preincubated with Nsp8, Nsp7/Nsp8 complex, Nsp12, and S protein, respectively, for 30 min before the RNA substrate was introduced. Wild-type Nsp15 and mutants D39A, E263A, and N157A were preincubated with 0.2 μM Nsp8 or 0.2 μM Nsp7/Nsp8 complex. The effects of divalent ions were detected in the presence of 0 mM, 2 mM, 5 mM, 10 mM, or 15 mM MnCl_2 or MgCl_2 . The enzymatic activity data were analyzed in Origin 8.0 (OriginLab). All assays were repeated three times, and the means $\pm$ SD are shown.

Binding affinity quantifications by MST. Binding affinity was detected by microscale thermophoresis (MST) using Monolith NT.115 (Nanotemper Technologies) as previously reported (24). Purified MERS-Nsp15 was labeled and centrifuged at 14,000 rpm for 10 min to eliminate precipitates. A serial dilution of recombinant Nsp7, Nsp8, Nsp12, and the Nsp7/Nsp8 complex was applied in buffer containing 20 mM Tris (pH 7.0), 50 mM NaCl, and 0.05% Tween 20. Affinity measurements were conducted in hydrophilic capillaries (K002 Monolith NT.115 hydrophobic capillaries; Nanotemper). Each measurement was repeated at least three times. The sigmoidal curves were normalized with the mean $\pm$ SD of each data point, and K_d values were calculated.

Pulldown assays and Western blotting. Purified Flag-tagged Nsp15 was incubated with His-tagged Nsp8, His-tagged Nsp8/Nsp7 (Nsp7 has no tag) complex, and His-tagged Nsp12 at 4°C overnight and then applied to anti-Flag beads. Nsp15 mutants N38A, D39A, N157A, and E263A were bound to anti-Flag beads and incubated with His-tagged Nsp8, with His-tagged Nsp12, or without protein. Beads were washed with phosphate-buffered saline (PBS) and resuspended in electrophoresis sample buffer for SDS-PAGE. The polyacrylamide gels were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% milk in Tris-buffered saline–Tween 20 (TBST). Anti-His antibodies were applied for 1 h at room temperature, and then the blots were washed with TBST buffer. After incubation with the second antibody, blots were washed, and proteins were detected and documented on X-ray film.

Accession number(s). The coordinates and structure factors for MERS-Nsp15 have been deposited in the Protein Data Bank (PDB) under accession code 5YVD.

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