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Variable Oligomerization Modes in Coronavirus Non-structural Protein 9

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Non-structural protein 9 (Nsp9) of coronaviruses is believed to bind single-stranded RNA in the viral replication complex. The crystal structure of Nsp9 of human coronavirus (HCoV) 229E reveals a novel disulfide-linked homodimer, which is very different from the previously reported Nsp9 dimer of SARS coronavirus. In contrast, the structure of the Cys69Ala mutant of HCoV-229E Nsp9 shows the same dimer organization as the SARS-CoV protein. In the crystal, the wild-type HCoV-229E protein forms a trimer of dimers, whereas the mutant and SARS-CoV Nsp9 are organized in rod-like polymers. Chemical cross-linking suggests similar modes of aggregation in solution. In zone-interference gel electrophoresis assays and surface plasmon resonance experiments, the HCoV-229E wild-type protein is found to bind oligonucleotides with relatively high affinity, whereas binding by the Cys69Ala and Cys69Ser mutants is observed only for the longest oligonucleotides. The corresponding mutations in SARS-CoV Nsp9 do not hamper nucleic acid binding. From the crystal structures, a model for single-stranded RNA binding by Nsp9 is deduced. We propose that both forms of the Nsp9 dimer are biologically relevant; the occurrence of the disulfide-bonded form may be correlated with oxidative stress induced in the host cell by the viral infection.

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Introduction

Since a coronavirus was identified as the causative agent of the 2003 outbreak of severe acute respiratory syndrome (SARS),[1–4] scientific interest in this family of viruses has increased dramatically.[5] Coronaviruses are enveloped, positive-strand RNA viruses that cause a wide spectrum of disease in humans and animals. These viruses are divided into three distinct groups on the basis of genome organization and phylogenetic analysis. Human coronavirus 229E (HCoV 229E) causes a mild form of the common cold and belongs to group 1, which includes the recently discovered human coronavirus NL63[6] and the porcine coronavirus, transmissible gastroenteritis virus (TGEV). Human coronaviruses belonging to group 2 are OC43 and HKU1, the latter also having been discovered very recently.[7] The SARS coronavirus has been classified as an outlier of group 2.[8] Coronaviruses infecting birds have been identified as a separate group (group 3).[9]

The genome of HCoV 229E consists of 27,277 nucleotides, comprising a total of eight open reading frames. The entire replicase complex of the virus is encoded within two large overlapping open reading frames, ORF 1a and ORF 1b. ORF 1a codes for

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Abbreviations used: SARS-CoV, severe acute respiratory syndrome coronavirus; HCoV-229E, human coronavirus 229E; Nsp, non-structural protein; MPD, 2-methyl-2,4-pentanediol; DLS, dynamic light-scattering; M^{pro}, main proteinase; r.m.s., root-mean-square; SSB, single-stranded DNA-binding protein; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; OB, oligonucleotide/oligosaccharide-binding; pp1a, polyprotein 1a; RU, resonance units.

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polyprotein 1a (pp1a) with a calculated molecular mass of 454 kDa. Involving a (−1) ribosomal frameshift, translation of ORF 1a and ORF 1b together yields the giant polypeptide 1ab (pp1ab) with a calculated molecular mass of 754 kDa.[10,11] These polyproteins are processed by two virus-encoded papain-like proteases ($PL1^{pro}$ and $PL2^{pro}$)[12] and the main proteinase (M^{pro}, also called 3C-like protease, $3CL^{pro}$),[13] resulting in 16 non-structural proteins (Nsps). The crystal structure of HCoV-229E M^{pro} has been determined by our group[14] and shown to be similar to that of the homologous enzyme from TGEV.[15] The structure of the SARS-CoV M^{pro}[16,17] is also very similar.

The C-terminal region of pp1a comprises a set of relatively small polypeptide domains, Nsp6–Nsp11. In preliminary experiments, we have shown that Nsp10 from mouse hepatitis (corona)virus (MHV) is a double-stranded RNA-binding zinc-finger protein,[18] and that HCoV-229E Nsp8 and Nsp9 interact with nucleic acids.[19] Also, it has been proposed recently that Nsp9 might interact specifically with the stem–loop II motif (s2m), a well defined RNA secondary-structure element at the 3′ end of many coronavirus genomes.[20] However, s2m does not seem to be conserved in HCoV 229E†. Nsp8 of SARS-CoV has the function of an RNA primase;[21] its 8:8 complex with Nsp7 has a three-dimensional structure reminiscent of the β_2 "sliding clamp" of bacterial DNA polymerase, with a central channel suitable for double-stranded RNA binding.[22] It has been shown by analytical ultracentrifugation that Nsp8 also interacts with Nsp9,[23] although according to our own measurements using surface plasmon resonance, this interaction is either absent or very weak (R.P., unpublished results). Colocalization of Nsp7, Nsp8, Nsp9, and Nsp10 was observed in MHV.[24] Very likely, these non-structural proteins are involved directly in the replication complex built around the RNA-dependent RNA polymerase (Nsp12).

Here, we describe the crystal structures of wild-type HCoV-229E Nsp9 at 1.75 Å resolution and its Cys69Ala mutant at 1.80 Å. In spite of 45% sequence identity between SARS-CoV and HCoV-229E Nsp9, the wild-type structure of the latter exhibits a mode of homodimerization that is entirely different from what has been observed in the crystal structure of the former.[23,25] To probe the effect of the observed intermolecular disulfide bridge on the formation of the HCoV-229E Nsp9 dimer, Cys69 was mutated to alanine. The crystal structure of this Nsp9 mutant shows a dimerization mode similar to that observed in SARS-CoV Nsp9.[23,25] However, gel mobility-shift assays and surface plasmon resonance (SPR) measurements indicate that only the wild-type HCoV-229E Nsp9, not the Cys69Ala mutant, binds strongly to single-stranded RNA and single-stranded DNA. In order to assess a possible direct role of Cys69 in nucleic acid binding, this residue was also replaced by serine. Again, the mutant showed little or no affinity to single-stranded DNA (ssDNA). Finally, the corresponding residue (Cys73) of SARS-CoV Nsp9, which did not form a disulfide bond, was replaced by alanine and serine. Both mutants showed wild-type affinity to single-stranded oligonucleotides. It is therefore concluded that Nsp9 of HCoV 229E is substantially different from its orthologue in SARS-CoV.

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Results

Structure elucidation and quality of the structural models

Wild-type HCoV-229E Nsp9 and its Cys69Ala and Cys69Ser mutants were cloned with a His_6 tag connected to the N terminus of the protein *via* the linker sequence VKLQ. The latter tetrapeptide corresponds to the C terminus of SARS-coronavirus Nsp8 (as well as HCoV-229E Nsp8) and therefore introduces a cleavage site for the main proteinase (M^{pro}) of SARS-CoV. After purification of the His_6-tagged protein using Ni-NTA chromatography, cleavage with the M^{pro} yielded Nsp9 with an authentic N terminus. The wild-type Nsp9 was crystallized using a reservoir containing 1.8–2.1 M ammonium sulfate, 0.1 M sodium acetate pH 4.0–4.5, and 5% (v/v) 2-methyl-2,4-pentanediol (MPD). Crystals were of space group *P*622, with a monomer in the asymmetric unit (Table 1). The structure was determined by molecular replacement, using a monomer of the SARS-CoV Nsp9[25] as the search model, and refined to 1.75 Å resolution. Residues 1–7 and 33–36 could not be modeled due to lack of electron density. Alternate conformations were detected in the electron density for the side-chains of Met9 and Lys82. The final *R*-factor for the structural model is 19.0% and the R_{free} is 22.4%; 97.2% of the amino acid residues are in the most-favored regions of the Ramachandran plot and the remainder in the additionally allowed regions.[26]

The Cys69Ala mutant of HCoV-229E Nsp9 was prepared by single-site PCR mutagenesis from the wild-type plasmid. Preparation of the protein was identical with wild-type Nsp9. The conditions identified for crystallization of the wild-type Nsp9 failed to yield crystals of the mutant. Instead, the following crystallization conditions were established: 0.2 M ammonium sulfate, 0.2 M sodium acetate (pH 4.6), 30% (w/v) polyethylene glycol monomethyl ether (PEG-MME) 2000. The crystals displayed space group $P2_12_12_1$, with a dimer of the Nsp9 mutant per asymmetric unit (Table 1). Residues 1, 2 and 109 of monomer A have not been modeled due to lack of electron density; the same is true for residues 1–4 and 107–109 of monomer B. The segment comprising residues 53–56 could be built into electron density but proved to be very flexible. The structure was refined to a resolution of 1.80 Å, with $R = 22.1\%$ and $R_{free} = 28.1\%$ (see Supplementary Data Fig. S1). Of the amino acid residues in the structural model,

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