The novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic known as COVID-19 (1–3), which can be prevented by vaccines but for which antiviral treatments are much needed. Coronaviruses employ a multisubunit machinery for replication and transcription. A set of nonstructural proteins (nsps) produced as cleavage products of the ORF1a and ORF1ab polyproteins (4) assemble to facilitate viral replication and transcription. The core component of this complex is the catalytic subunit (nsp12) of an RNA-dependent RNA polymerase (RdRp) (5), which catalyzes the synthesis of viral RNA and thus plays a central role in the replication and transcription cycle of SARS-CoV-2, with the assistance of nsp7 and nsp8 as accessory factors (6, 7). Structures of the RdRp (nsp12-nsp7-nsp8 complex) alone and in complex with the helicase have been determined by cryo–electron microscopy (cryo-EM) (8–11); in all of these structures, the RdRp of SARS-CoV-2 was proposed to contain zinc ions ligated in the same locations as those observed in SARS-CoV (7) in highly conserved metal binding motifs composed of H295-C301-C306-C310 and C487-C551 (locations as those observed in SARS-CoV (7)) and characterized by electron microscopy structures of the RdRp complex. These metal binding sites are essential for replication and for interaction with the viral helicase. Oxidation of the clusters by the stable nitroxide TEMPOL caused their disassembly, potently inhibited the RdRp, and blocked SARS-CoV-2 replication in cell culture. These iron-sulfur clusters thus serve as cofactors for the SARS-CoV-2 RdRp and are targets for therapy of COVID-19.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causal agent of COVID-19, uses the RNA-dependent RNA polymerase (RdRp) for the replication of its genome and the transcription of its genes. We found that the catalytic subunit of the RdRp, nsp12, ligates two iron-sulfur metal cofactors in sites that were modeled as zinc centers in the available cryo–electron microscopy structures of the RdRp complex. These metal binding sites are essential for replication and for interaction with the viral helicase. Oxidation of the clusters by the stable nitroxide TEMPOL caused their disassembly, potently inhibited the RdRp, and blocked SARS-CoV-2 replication in cell culture. These iron-sulfur clusters thus serve as cofactors for the SARS-CoV-2 RdRp and are targets for therapy of COVID-19.

Having recently demonstrated that we are able to predict the presence of Fe-S cofactors in candidate proteins based on the identification of specific amino acid sequence motifs (27), we analyzed the primary sequences of SARS-CoV-2 proteins to investigate whether any might incorporate Fe-S clusters. We identified two highly conserved Lyr (leucine-arginine-tyrosine)–like motifs (fig. S2A) in nsp12 that have been previously characterized as potential binding sites for the cochaperone HSC20 (also known as HSCB) of the Fe-S biogenesis machinery (27–30), which facilitates Fe-S cluster transfer from the main scaffold protein, ISCU (iron-sulfur cluster assembly scaffold), to recipient proteins (fig. S2B). To assess whether the Lyr-like motifs were involved in direct binding of nsp12 to HSC20, we incubated full-length SARS-CoV-2 nsp12 wild type (WT) or variants wherein either or both Lyr motifs were replaced by alanines (A) (fig. S2C) with purified HSC20. Nsp12 WT bound HSC20, indicating that the RdRp subunit interacts directly with the cochaperone (fig. 1A). Substitution of either of the two Lyr motifs with alanines decreased the amount of bound HSC20 (fig. 1A), which was even more profoundly diminished by loss of both motifs in nsp12VYR/LYR-AAA (fig. 1A). Coimmunoprecipitation (co-IP) experiments in Vero E6 cells and mass spectrometry analysis confirmed that nsp12 transiently interacted with HSC20 and with components of the de novo Fe-S cluster (the chaperone HSPA9, the cysteine desulfurase NFS1, and the main scaffold ISCU) and cytoplasmic Fe-S (CIA) biogenesis (CIA01, MMS19, and FAM96B) machineries (fig. 1B and C; fig. S2D; and data S1), suggesting that these interactions may be required for Fe-S cluster acquisition by nsp12. To investigate whether nsp12 coordinated an Fe-S cluster, we quantified $^{57}$Fe incorporation into the protein expressed in cells transfected with either a pool of nontargeting small interfering RNAs (NT siRNAs) or with siRNAs against the initial Fe-S biogenesis scaffold, ISCU. In control cells (NT siRNAs), nsp12 WT bound radiolabeled iron (8312 ± 775 cpm/mg of cytosolic proteins) (fig. 1, D and E), whereas nsp12 that lacked the Lyr motifs did not interact with HSC20 and bound significantly less iron (250 ± 92 cpm/mg of cytosolic proteins) (fig. 1, D and E). Nsp12 expressed in cells silenced for ISCU (si-ISCU) failed to incorporate iron (fig. 1, D and E). Taken together, these results demonstrate that nsp12 binds iron, likely in the form of an Fe-S cluster. Nsp12 expressed in Expi293F mammalian cells and purified axially oxidized a shoulder at ~420 nm in its ultraviolet–visible (UV-vis) absorption spectrum (fig. 2, A and B, and fig. S3, A and B), suggesting that it harbored one or more Fe-S clusters (31, 32). To determine the type and stoichiometry of the Fe-S cluster(s), a $^{57}$Fe-enriched nsp12-FLAG sample was analyzed by Mössbauer spectroscopy (fig. 2C). The 4.2-K Mössbauer spectrum collected in a 53-mT magnetic field applied parallel to the direction of gamma radiation (fig. 2C) shows the presence of a single quadrupole doublet with parameters typical of [Fe$_4$S$_4$]$^{2+}$ clusters (isomer shift (δ) of 0.44 mm/s and quadrupole splitting parameter (ΔEQQ) of 1.25 mm/s, blue line) (%) wild-type nsp12 bound 7.5 ± 0.35 iron atoms per monomer, and we thus interpret the Mössbauer spectrum as two [Fe$_4$S$_4$]$^{2+}$ clusters. The X-band electron paramagnetic resonance (EPR) spectrum, recorded at 20 K, showed no signal (fig. S3C), ruling out the presence of Fe-S clusters with a half-integer spin ground state. However, upon reduction with dithionite, EPR signal characteristics of [Fe$_4$S$_4$]$^{2+}$ clusters were observed (fig. S3D) (34). Notably, the nsp12-nsp7-nsp8 complex anoxically purified with the Fe-S cluster(s) showed markedly increased binding to the template and RNA primer (fig. S4) and increased polymerase activity relative to the aerobically purified complex that contained two zinc ions per protomer (fig. 2D and fig. S4). The available cryo-EM structures of the RdRp complex have assigned two chelated zinc ions in the highly conserved metal binding motifs of nsp12 composed of H295-C301-C306-C310 at the interface between the NIRAN (nidovirus RdRp–associated nucleotidyltransferase) domain and
the catalytic domain and of C487-H642-C645-C646 in the fingers of the catalytic domain (7–11) (Fig. 2E and fig. S1; see supplementary text). By replacing selected cysteines with serines and characterizing the variant nsp12 proteins, we tested the hypothesis that the two [Fe₄S₄] clusters are coordinated by these motifs. The two variants lacking any one of the set of three Cys residues of either the interfacial motif (nsp12C301S-C306S-C310S) or the catalytic domain (nsp12 C487S-C645S-C646S) (replaced by Ser) contained 3.8 ± 0.2 and 3.67 ± 0.3 Fe per nsp12 protomer, respectively, and exhibited approximately half of the absorbance at 420 nm (Fig. 2A and B, and fig. S3, A and B).
Fe radiolabel seen for the WT nsp12 (Fig. 2F).

The 4.2-K/53-mT Mössbauer spectra of these two variants revealed that ~95% of Fe is associated with the quadrupole doublet with the same parameters deduced from the spectrum of WT nsp12, thus revealing the presence of one [Fe4S4]2+ cluster in the unmodified binding site (Fig. 2C). The 20-K X-band EPR spectra of the variants after they were treated with sodium dithionite are also consistent with the presence of one [Fe4S4]2+ cluster (fig. S3D). A variant lacking a total of four cysteines from both motifs (nsp12C301S-C306S-C645S-C646S) did not bind Fe and had no absorbance at 420 nm, consistent with the notion that both [Fe4S4]c l u s t e rb i n d i n g sites had been eliminated (Fig. 2, A and B, and fig. S3, A and B). The two [Fe4S4] j 3+ clusters incorporated in a mammalian overexpression system are thus ligated by cysteine residues located in the two zinc-binding sites identified in the cryo-EM structures.

We next aimed to characterize the role of the two Fe-S clusters in the RdRp. Functional studies revealed that the [Fe4S4] cluster in the catalytic domain of nsp12 is required for the RNA polymerase activity of the nsp12-nsp7-nsp8 complex (Fig. 3, A and B), in addition to presumably maintaining structure. In fact, the absence of the cysteine ligands in the catalytic domain in the nsp12C487S-C645S-C646S variant caused a more profound decrease in the polymerase activity than was observed in the zinc complex (Fig. 3A), suggesting that Zn, by coordinating the same cysteine residues, can partially fulfill the structural role of the Fe-S cluster, preserve the architecture of the fingers subdomain, and maintain some polymerase activity, which is strictly associated with the palm of the catalytic domain (8, 11). Fe-S enzymes involved in DNA and RNA metabolism have often been mischaracterized as zinc-containing proteins, as Fe-S clusters readily undergo oxidative degradation during standard aerobic purification procedures of proteins, allowing zinc to coordinate the same cysteine residues.

55Fe incorporation into nsp12 WT or variants

***P < 0.001.
Moreover, zinc-containing enzymes have been shown to retain activity in vitro on short templates (14, 35), which previously supported the conclusion that zinc was the physiological cofactor of these enzymes. Fe-S clusters in nucleic acid metabolism enzymes have been thought to participate directly not in catalysis but in modulating binding of the enzyme to the template and/or to other components of the replication complex (26, 36, 37), as well as in increasing processivity and enabling repair through a proposed charge-transfer mechanism (38, 39). Consistent with the notion that zinc is likely not the physiological cofactor in several viral replicases that have so far been crystallized with chelated zinc ions, supplementation with zinc has been reported to inhibit replication in several cell culture models of viral infection (40–42). Loss of the [Fe₄S₄] cluster ligated by H295-C301-C306-C310, which is located at the interface between the NiRAN and the catalytic domain of nsp12, had minimal effect on the RNA polymerase activity (Fig. 3, A and B). However, loss of this cluster profoundly diminished the interaction with the helicase nsp13 (Fig. 3, B and C), which is an essential component of the replication complex.

We attempted to exploit the sensitivity of Fe-S clusters to oxidative degradation (43) to prevent coronavirus replication in cell culture models. Previous studies have shown that a stable nitroxide, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), was beneficial in two different animal models of human conditions through its ability to oxidize and disassemble the Fe-S cluster of cytosolic aconitate (IRP1), thereby converting it into the iron responsive element (IRE)–binding apo-form (44–46). RdRp isolated from Expi293F cells that had been treated with TEMPOL (Fig. 4A) had diminished absorbance at 420 nm relative to the complex isolated from untreated cells, indicative of loss of the Fe-S clusters of nsp12. Likewise, treatment with TEMPOL of the Fe-S cluster–containing protein in vitro caused loss of absorbance in the same region (Fig. 4B). Either treatment resulted in loss of polymerase activity (Fig. 4, C to E). The TEMPOL treatment of cells did not impact the activities of several mitochondrial Fe-S enzymes, including the respiratory complexes and mitochondrial aconitate (ACO2), and the cytosolic Fe-S enzyme dihydroyperimidine dehydrogenase (DPYD) (figs. S5 and S6, A to F), nor did it cause any cytotoxicity at doses up to 5 mM (fig. S6G). TEMPOL treatment also did not affect the interactions of nsp12 with the components of the Fe-S and CIA biogenesis machinery from which nsp12 acquires its Fe-S clusters (fig. S7). We thus infer that TEMPOL directly reacts with Fe-S clusters in RdRp, leading to their degradation.

In support of this mechanism of action, diethylamine nonoate (DEA/NO), a nitric oxide donor that readily reacts with Fe-S clusters to form dinitrosyl complexes with diminished absorbance (47, 48), also inhibited the RdRp (Fig. 4E and fig. S8), although less effectively than TEMPOL. We found that TEMPOL was both a more potent RdRp inhibitor (fig. S9) and synergized with remdesivir (RDV) (fig. S10), a nucleoside analog that has been used to target the replication of SARS-CoV-2 (49). RDV was notably less effective against the Fe-S–RdRp than the zinc–RdRp (fig. S11).

Having demonstrated a strong inhibitory effect of TEMPOL on the activity of the RdRp of SARS-CoV-2, we asked whether TEMPO might exhibit antiviral activity against live virus replication. Vero E6 cells were infected with the SARS-CoV-2 USA-WA1/2020 isolate...
in the presence of increasing concentrations of TEMPOL (range: 0.1 to 1 mM). TEMPOL exhibited strong antiviral activity at concentrations above 0.2 mM. Viral titers were reduced by more than 5 log10 in the presence of 0.4 mM TEMPOL, which is reported to have a 50% cytotoxic concentration (CC50) greater than 100 mM ([2][3]). Our studies present a molecular basis for pursuing TEMPOL—with its low cytotoxicity and known access to tissues relevant for COVID-19 infection ([51][52])—and other related stable nitroxides as potential SARS-CoV-2 therapies during active viral infection.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

Figs. S1 to S11

References (54–66)

MDAR Reproducibility Checklist

Data S1

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