Molecular Basis for ADP-Ribose Binding to the Mac1 Domain of SARS-CoV-2 nsp3

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ABSTRACT: The virus that causes COVID-19, SARS-CoV-2, has a large RNA genome that encodes numerous proteins that might be targets for antiviral drugs. Some of these proteins, such as the RNA-dependent RNA polymerase, helicase, and main protease, are well conserved between SARS-CoV-2 and the original SARS virus, but several others are not. This study examines one of the proteins encoded by SARS-CoV-2 that is most different, a macrodomain of nonstructural protein 3 (nsp3). Although 26% of the amino acids in this SARS-CoV-2 macrodomain differ from those observed in other coronaviruses, biochemical and structural data reveal that the protein retains the ability to bind ADP-ribose, which is an important characteristic of beta coronaviruses and a potential therapeutic target.

The development of antivirals targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the present COVID-19 pandemic, will most likely focus on viral proteins and enzymes needed for replication. Similar to other coronaviruses, SARS-CoV-2 has a large positive sense (+)RNA genome that is more than 30000 nucleotides long with several open reading frames. Most of the proteins that form the viral replicase are encoded by the "rep 1ab" reading frame, which encodes a 7096-amino acid polyprotein that is ultimately processed into at least 15 functional peptides, five of which are produced only by a translational frameshift event occurring after nsp10 (Figure 1). Parts of the SARS-CoV-2 rep 1ab polyprotein are very similar to the rep 1ab protein of the coronavirus that caused the SARS epidemic in 2003 (which here will be termed SARS-CoV), suggesting that the drugs targeting SARS-CoV nsp5–14 might be effective against SARS-CoV-2. However, some portions of the SARS rep 1ab polyproteins are quite different.

In contrast to the well-conserved SARS-CoV nsp5 protease, nsp12 polymerase, and nsp13 helicase enzymes, significantly more differences exist between the nsp3 proteins encoded by SARS-CoV and SARS-CoV-2. The most variation occurs in a domain of nsp3 suspected to bind ADP-ribose, which will here be termed the Mac1 domain, to differentiate it from the two downstream macrodomains (Mac2 and Mac3), which do not bind ADP-ribose. The Mac1 domain of SARS-CoV also catalyzes the hydrolysis of ADP-ribose 1″ phosphate, albeit at a slow rate. Some viral macrodomains also remove ADP-ribose from proteins, and this de-ADP-ribosylation activity correlates with virulence and the ability to evade the innate immune response. Jean-Michel Claverie recently suggested that the putative ability of Mac1 to remove ADP-ribose from proteins might be related to the cytokine storm syndrome seen in severe cases of COVID-19.

Compounds blocking ADP-ribose binding could be used to test this important hypothesis. However, the many sequence differences preclude the use of the SARS-CoV Mac1 domain structures as scaffolds to design compounds that might target this nsp3 region in SARS-CoV-2. ADP-ribose binding must also be confirmed, especially in light of the observation that the same nsp3 domain from gamma coronaviruses does not bind ADP-ribose in vitro. The ability of the SARS-CoV-2 Mac1 domain to bind ADP-ribose was therefore examined here using a recombinant purified protein and isothermal titration calorimetry (ITC). We also determined the structure of the SARS-CoV-2 Mac1 domain to examine the biochemical context of ADP-ribose binding and to provide data for rational inhibitor design or in silico screening.

MATERIALS AND METHODS

Gene Synthesis. To facilitate the comparison between SARS-CoV and SARS-CoV-2, a protein expression vector was generated that is similar to that used by Eglott et al. To this

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end, a codon-optimized open reading frame was synthesized by GenScript (Piscataway, NJ) that encodes the Mac1 domain with an N-terminal TEV-cleavage site flanked by NheI and BamHI restriction sites. This open reading frame was cloned into pET21b to yield plasmid pET21-COVID-Mac1. The pET11-COVID-Mac1 plasmid was used to transform BL21(DE3) cells.

**Protein Purification.** Colonies of BL21(DE3) cells harboring the pET21-COVID-Mac1 plasmid were used to inoculate 3 mL of lysogeny broth containing 100 mg/mL ampicillin. The starter culture was incubated at 37 °C while being shaken at 225 rpm. After the cells grew to an OD600 of 1.0, they were transferred to 1 L of fresh medium containing ampicillin. After the cells reached an OD600 of 1.0 again, protein production was induced with 1 mM isopropyl β-D-thiogalactoside. After growing for 16 h at 23 °C, cells were harvested by centrifugation at 4000 rpm and 4 °C. The resulting cell pellet was suspended in 25 mL of IMAC buffer [20 mM Tris (pH 8) and 0.5 M NaCl], sonicated on ice for five 1 min bursts separated by 2 min rests, and clarified by centrifugation at 10000g for 30 min. The supernatant was loaded onto a 5 mL Ni-NTA column, and the fractions were eluted with a step gradient from 5 to 500 mM imidazole. Fractions containing the Mac1 domain protein (5 mL total) were loaded on a 250 mL Sephacryl S300 gel filtration column and eluted with 10 mM MOPS and 150 mM NaCl. Purified protein concentrations were determined by measuring the absorbance at 260 nm using a molar extinction coefficient of 10555 M⁻¹ cm⁻¹, which was calculated using the ProtParam tool (https://web.expasy.org/protparam/).

**Isothermal Titration Calorimetry (ITC).** Binding of ADP-ribose to the SARS-CoV-2 Mac1 domain was measured using a Nano ITC apparatus (TA Instruments). Before the measurement was started, samples of both ligand and protein were diluted in 10 mM MOPS and 150 mM NaCl (pH 7) and degassed at 400 mmHg for 30 min. Measurements were taken at 20 °C by injecting 2.0 μL aliquots of 500 μM ADP-ribose (Sigma) into 50 μM protein (175 μL initial volume) with stirring at 250 rpm. Using NanoAnalyze Software (version 3.11.0), data were fitted by nonlinear regression to an independent binding model. Briefly, after baseline correction, background heats from ligand-to-buffer titrations were subtracted, and the corrected heats from the binding reaction were used to identify best fit parameters for the stoichiometry of binding (n), free energy of binding (ΔG), apparent enthalpy of binding (ΔH), and entropy change (ΔS). Dissociation constants (Kd) were calculated from the ΔG.

**Crystallization and Structure Determination.** In preparation for crystallization experiments, purified SARS-CoV-2 Mac1 domain protein was cleaved with tobacco etch virus (TEV) protease to remove the N-terminal His tag and passed back through the Ni-NTA column. The flow-through fractions were desalted into 10 mM HEPES (pH 7.2) using a...
2 × 5 mL HiTrap desalting column (GE Life Sciences) and concentrated to 10 mg/mL in a centrifugal concentrator. This preparation of the protein was mixed at a 1:1 μL ratio with Morpheus HT screen reagents (Molecular Dimensions) in a 96-well SwissSci MRC ultraviolet-transmissible sitting drop plate. Large, diffraction-quality crystals grew directly from a number of the screen conditions. The crystal ultimately used for structure determination grew from condition D9: 0.12 M alcohols (0.02 M each 1,6-hexanediol, 1-butanol, 1,2-propanediol, 2-propanol, 1,4-butanediol, and 1,3-propanediol), 0.1 M buffer system 3, pH 8.5 (0.05 M Tris and 0.05 M bicine), and 30% precipitant mix 1 [20% poly(ethylene glycol) (PEG) 500 monomethylether and 10% PEG 20000]. Large, thick plates grew within 1 week at 22 °C. Given the high concentration of PEG 500 MME, the crystal did not require additional cryo-protection and was flash-cooled by being directly looped from the sitting drop and plunged into liquid nitrogen.

Diffraction data were collected on Life Sciences Collaborative Access Team (LS-CAT) beamline 21-ID-F at the Advanced Photon Source of Argonne National Laboratory. The wavelength at this station is fixed at 0.9787 Å; the detector is a MarMosaic M300 charge-coupled device. The data were collected with an oscillation width of 0.5° per image for a total oscillation of 180°. The data were indexed and integrated with DIALS15,16 as implemented in version 7.2 of the CCP4 software suite.17,18 Data scaling and reduction were performed using AIMLESS. Values in parentheses apply to the high-resolution shell indicated in Table 1. The coordinates were deposited in the Protein Data Bank as entry 6WYE.

## RESULTS AND DISCUSSION

### Variability in the nsp3 Mac1 Domain

The structures of most of the soluble portions of the SARS-CoV nsps were examined at atomic resolution to help understand coronavirus replication and facilitate antiviral drug discovery. The amino acid sequences of each of these proteins were compared with the homologous regions of the rep 1ab protein encoded by SARS-CoV-2 (GenPept accession number YP_009724389). The most similar proteins were the RNA helicases (nsp13), which are identical in all but one of their 603 amino acids, namely a conservative Val to Ile substitution near their C-termini. The RNA-dependent RNA polymerases (nsp12) are also well conserved, sharing all but 34 of 955 amino acids. The primary protein that cleaves the polyprotein (nsp5) is also similar in SARS-CoV and SARS-CoV-2, with only 13 amino acids that differ among 306 (4.2% different) (Figure 1).

At the other end of the spectrum are the nsp3 proteins, which are notably more different in the two SARS viruses. nsp3 is a large multidomain membrane-bound protein, and its clearest role in viral replication is cleaving the rep polyprotein. Greater than 17% of the amino acids in the nsp3 protease domain differ between SARS-CoV and SARS-CoV-2.

![Table 1: Crystallographic Data Collection and Model Refinement Statistics for the SARS-CoV-2 Mac1 Domain](https://dx.doi.org/10.1021/acs.biochem.0c00309)

| Data Collection |  |
|-----------------|-------------------|
| resolution range (Å) | 43.32—0.95 (0.97—0.95) |
| space group | P2₁,2₁,2₁ |
| a, b, c (Å) | 43.3, 54.4, 67.6 |
| α, β, γ (deg) | 90.0, 90.0, 90.0 |
| Rmerge | 0.063 (0.348) |
| Rmerge | 0.075 (0.475) |
| CC1/2 | 0.039 (0.320) |
| CC1/2 | 99.5 (89.1) |
| no. of unique reflections | 99442 (4199) |
| completeness (%) | 98.4 (85.0) |
| multiplicity | 6.4 (2.6) |
| (λ/σ(λ)) | 13.1 (1.5) |

Model Refinement

|  |
|-----------------|-------------------|
| no. of reflections used in refinement | 99335 (2606) |
| nRF of reflections used for Rfree | 5046 (125) |
| Rmerge (Rfree) | 0.119 (0.137) |
| Wilson B-factor (Å²) | 7.4 |
| average B-factor (Å²) | 13.9 |
| protein atoms | 10.2 |
| solvent | 28.2 |
| root-mean-square (RMS) deviation | 0.010 |
| bond lengths (Å) | 1.16 |
| bond angles (deg) | 0.06 |
| coordinate error (Å) | 98.2/1.8/0.0 |
| Ramachandran statistics | 1.2 |
| favored/allowed/outliers (%) | 98.2/1.8/0.0 |
| rotamer outliers (%) | 1.2 |
| Clashscore | 1.70 |

Other parts of nsp3 are even more variable, such as the macrodomains that lie N-terminal to the nsp3 protease domain. Macrodomains consist of four helices that surround a mixed β sheet. A ligand-binding pocket that typically binds ADP-ribose or related compounds lies between the helices and the sheet. SARS-CoV has three macrodomains in tandem, but only the first binds ADP-ribose. The amino acid sequences of this Mac1 domain differ by 26% between SARS-CoV and SARS-CoV-2 (Figure 1).

Six of the 47 variant residues in the 180-amino acid SARS Mac1 domain are clustered in a 21-amino acid region near its N-terminus, which is particularly variable in the three Coronaviridae genera (Figure 2) and sometimes not included in other macrodomains. As shown by the conservation plot above the alignment (Figure 2), there are several highly variable regions throughout the protein, which might affect protein function. Mac1 domains from coronaviruses that cause the common cold (alpha coronaviruses) and the beta respiratory syndrome coronavirus (MERS-CoV), all bind ADP-ribose. However, reports with gamma coronaviruses suggest that ADP-ribose binding might not be conserved. Sequence differences exist between infectious bronchitis virus (IBV) strain M41, which binds ADP-ribose, and IBV strain Beaudette, which does not.

### Expression and Purification of the SARS-CoV-2 Mac1 Domain

An *Escherichia coli* expression vector for the Mac1
domain was generated to express an N-terminally His-tagged protein similar to a SARS-CoV protein studied by Egloff et al.14 Upon induction, a 1 L culture of BL21(DE3) cells harboring the vector expresses 50−100 mg of the Mac1 domain protein that can be purified in one step to apparent homogeneity using immobilized metal affinity chromatography (Figure 3A). The protein was polished further with gel filtration chromatography and concentrated before analysis and crystallization.

Nucleotide Binding by the SARS-CoV-2 Mac1 Domain. Repeated ITC experiments revealed that the purified recombinant protein bound ADP-ribose (Figure 3C) with a dissociation constant of 10 ± 4 μM (the uncertainty is the standard deviation of $K_d$ from independent titrations). To examine binding specificity, similar titrations were repeated with related nucleotides. The SARS-CoV-2 protein bound ADP, CAMP, ATP, and ADP-glucose (Figure 3D). All nucleotides lacking the ribose moiety bound with similar high affinities, but none bound with an enthalpy change similar to that observed with ADP-ribose, suggesting that the ribose moiety becomes structured when bound to the macrodomain.

The energetics of binding of ADP-ribose to the SARS-CoV protein are similar to those noted for the same protein from SARS-CoV14 and MERS-CoV.39 The enthalpy and entropy of binding were also very similar for all three proteins (Figure 3D). In contrast to findings from the Mac1 protein from an alpha coronavirus,13 enthalpy appears to drive binding of ADP-ribose to the Mac1 domains of the three beta coronaviruses. Variation in the ADP-ribose-binding cleft might account for these differences. For example, nsp3 amino acid 360, which is near the adenine base, is a Phe in SARS-CoV-2, an Asn in both SARS-CoV and MERS-CoV, and an aliphatic amino acid in the alpha and gamma coronaviruses (Figure 2).

Structure of the SARS-CoV-2 Mac1 Domain. The SARS-CoV-2 Mac1 domain (nsp3 residues 207−277) crystallized in space group $P2_12_12_1$ with one molecule per asymmetric unit. These crystals had a solvent content of 43% and diffracted extremely well. The final resolution limit of the data was set at 0.95 Å (Table 1). The quality of the electron density maps is correspondingly excellent (Figure 4A). The section of the structure depicted in this image is located on the surface of the protein, and the $B$-factors of these residues (7.2 Å$^2$) are close to the average $B$-factor of the protein (10.2 Å$^2$), indicating that this sample accurately represents the structure.
overall quality of the maps. The final model contains the entire sequence from V207 to S377 of nsp3, an N-terminal glycine residue that was left from the TEV-protease cleavage, and 374 solvent molecules. The $R_{\text{cryst}}$ and $R_{\text{free}}$ values of the final model were 0.119 and 0.137, respectively (Table 1).

As expected, the tertiary structure ranges from approximately identical to very similar to those of other coronavirus macrodomains, including SARS CoV (2FAV, 74.7% sequence identity) with a root-mean-square deviation (RMSD) value of 162 of 172 $\alpha$-atoms of 0.6 Å, MERS-CoV (5DUS, 42.2% identical) with a 1.2 Å RMSD for 161 of 172 $\alpha$-atoms, human alpha coronavirus 229E (3EWR, 41.2% identical) with a 1.2 Å RMSD for 161 of 172 $\alpha$-atoms, human alpha coronavirus (3JZT, 35.5% identical) with a 1.5 Å RMSD for 154 of 172 $\alpha$-atoms, feline coronavirus (FCoV, 3JZT, 26.8% identical) with a 1.5 Å RMSD for 153 of 172 $\alpha$-atoms, and the gamma CoV IBV (3EPW, 26.7% identical) with a 2.1 Å RMSD for 150 of 172 $\alpha$-atoms. The regions with a high degree of sequence conservation are not clustered in any particular region(s) of the molecule, as is clear when the ribbon is colored as a gradient from red (poorly conserved) to blue (highly conserved) (Figure 4B). This finding is consistent with the fact that the protein atoms involved in hydrogen bonding interactions with the ligands in these structures are more often part of the main chain; relatively few interactions of side chains with ligands are observed.

At the time of writing, we discovered that Michalska et al. of the Center for Structural Genomics of Infectious Diseases (CSGID) deposited coordinates for a very similar construct of the SARS-CoV-2 Mac1 domain including the region from E206 to E275 of the nsp3 protein plus an additional four residues at the N-terminus (6VXU, unpublished). Their crystals also allowed binding of ADP-ribose (6W02) and AMP (6W6Y), whereas ours seemed to be packed too tightly to permit ligands to access the binding site (data not shown). We compared our ultra-high-resolution model of the unliganded protein to the ADP-ribose-bound form. The RMSD for the fitting, which were determined by secondary structure matching (SSM) as implemented in COOT, is 0.59 Å for 165 of 172 Ca atoms. This value is very similar to the RMSD values of the free protein (6VXS, 0.66 Å) and the AMP-bound form (6W6Y, 0.50 Å), indicating that no large conformational changes occur upon ligand binding. In fact, the only notable conformational changes occur in three surface-exposed loops in or near the ligand-binding pocket (Figure 4C). These loops connect strand $\beta_2$ with helix $\alpha_2$ (the $\beta_2$–$\alpha_2$ loop), strand $\beta_4$ with helix $\alpha_4$ (the $\beta_4$–$\alpha_4$ loop), and strand $\beta_5$ with helix $\alpha_5$ (the $\beta_5$–$\alpha_5$ loop). The subtle change in conformation of the $\beta_4$–$\alpha_4$ loop (purple in Figure 4C) appears to be the result of crystal contacts and not the direct influence of ADP-ribose binding. The other two loops are more intimately involved in ligand binding. The main chain of the $\alpha_2$–$\beta_2$ loop rotates 180° to allow the amide N atom of G252 to participate in a hydrogen bonding interaction with the 1′-hydroxyl of the ribose moiety of ADP-ribose. G252 corresponds to residue V33 in the Chikungunya macrodomain, which Ecke et al. proposed is needed for de-ADP-ribosylation activity based on results of site-directed mutagenesis. This loop also carries N244, which directly interacts with the ribose. The phenyl ring of F336 in the $\beta_5$–$\alpha_5$ loop occupies the portion of the binding pocket in the unliganded structure that is occupied by the 1′-phosphate of ADP-ribose. Thus, without rearrangement of the $\beta_5$–$\alpha_5$ loop, ADP-ribose would not be able to bind.

**Figure 3.** SARS-CoV-2 Mac1 domain binds ADP ribose. (A) A 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel showing 10 µL samples of a soluble crude lysate of induced BL21(DE3) cells harboring plasmid p21-COVID-Mac1 (lane C), proteins that do not bind a Ni-NTA column (F), and fractions eluted from a Ni-nitrilotriacetic acid column during an imidazole step gradient from 0 mM (lanes 1–3) to 5 mM (lanes 4–6), 40 mM (lanes 7–9), and 500 mM (lanes 10–12). Protein markers (lane M) are 116, 66.2, 45, 35, and 25 kDa. (B) Example ITC experiment in which the purified SARS-CoV-2 nsp3 macrodomain was titrated with ADP ribose. (C) ITC experiments such as those shown in panel B were repeated thrice with each of the compounds listed. Means are plotted, and error bars are standard deviations. Average (±SD) dissociation constants were $10^{-4}$ µM for ADP-ribose, $3 \pm 3$ µM for ATP, $6 \pm 4$ µM for ADP-glucose, $2 \pm 1$ µM for cAMP, and $2 \pm 1$ µM for adenosine. (D) Comparison of the thermodynamics of ADP-ribose binding by Mac1 domains from SARS-CoV-2 (data from panel C), SARS-CoV, MERS-CoV, and an alpha coronavirus. *Data from ref 34 bData from ref 39. bData from ref 13.

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The significance of the study stems mainly from the demonstration that the SARS-CoV-2 Mac1 domain binds ADP-ribose. Although SARS-CoV and SARS-CoV-2 have 26% divergence in amino acid sequences, their structures are highly similar, which may explain the ability of the latter to bind ADP-ribose. This is the first step needed to justify screens for potential antivirals that bind in place of ADP-ribose. However, more work needs to be done to understand the antiviral potential of such compounds because the biological role for ADP-ribose binding is not completely understood.
understood. Some work with alpha coronaviruses suggests that ADP-ribose binding by the Mac1 domain is not required for viral replication. However, studies with other (+)RNA viruses suggest that macrodomains are essential for virulence. This work is also noteworthy because the synthetic codon-optimized plasmid reported here produces up to 100 mg of soluble Mac1 domain protein per liter of E. coli culture, and this protein retains a high affinity for ADP-ribose. The protein could be used for structural studies and screening campaigns. Screening assays with the SARS-CoV-2 protein might be more efficient because the SARS-CoV-2 protein binds ADP-ribose somewhat more tightly ($K_d = 10 \mu M$) than the SARS-CoV protein ($K_d = 24 \mu M$). The recombinant protein reported here together with detailed structural information might also be useful to others developing SARS-CoV-2 diagnostics and/or therapeutics.

**ASSOCIATED CONTENT**

Accession Codes
SARS-CoV-2 Rep 1ab, YP_009724389 (NCBI); SARS-CoV-2 Mac1, 6WEY (PDB).

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