

RESEARCH ARTICLE

Structure–function mapping and mechanistic insights on the SARS CoV2 Nsp1

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Abstract

Non-structural protein 1 (Nsp1) is a key component of the infectious process caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2), responsible for the COVID-19 pandemic. Our previous data demonstrated that Nsp1 can degrade both RNA and DNA in the absence of the ribosome, a process dependent on the metal ions Mn^{2+} , Ca^{2+} , or Mg^{2+} (Salgueiro et al., SARS-CoV2 Nsp1 is a metal-dependent DNA and RNA endonuclease. *Biomaterials*. 2024;37:1127–1146). The protein is composed of two structural domains: the N-terminal domain (NTD) and C-terminal domain (CTD), connected by a loop. To elucidate the function of each structural domain, we generated four truncated versions of Nsp1 containing either the NTD or the CTD. Our results indicate that the Nsp1^{SARS-CoV2} domains play distinct functional roles. Specifically, the NTD is involved in nucleotide binding and regulation, while the CTD acts as the catalytic domain. Moreover, a tyrosyl radical was detected during the nuclease activity, and an in-depth analysis of the different constructs suggested that Y136 could be involved in this process. Indeed, our results show that Y136F Nsp1 variant lacks DNA nuclease activity but retains its RNA nuclease activity. Furthermore, we observed that the CTD has a propensity to associate with hydrophobic environments, suggesting that it might associate with cell membranes. However, the cellular function of this association requires further investigation. This study sheds light on the functions of the individual domains of Nsp1, providing valuable insights into its mechanism of action in Coronaviruses.

KEYWORDS

biophysical characterization, fluorescence spectroscopy, metals, nuclease, SDS, tyrosyl radical

1 | INTRODUCTION

SARS-CoV2 is a member of the coronavirus family with an enveloped single-stranded RNA genome of about

30 kb (de Wit et al. 2016; Ksiazek et al. 2003; Lai et al. 2020; Zaki et al. 2012). Among its 16 non-structural proteins (Nsps) encoded by the viral genome, Nsp1 is exclusive to α and β coronaviruses (Gordon et al. 2020;

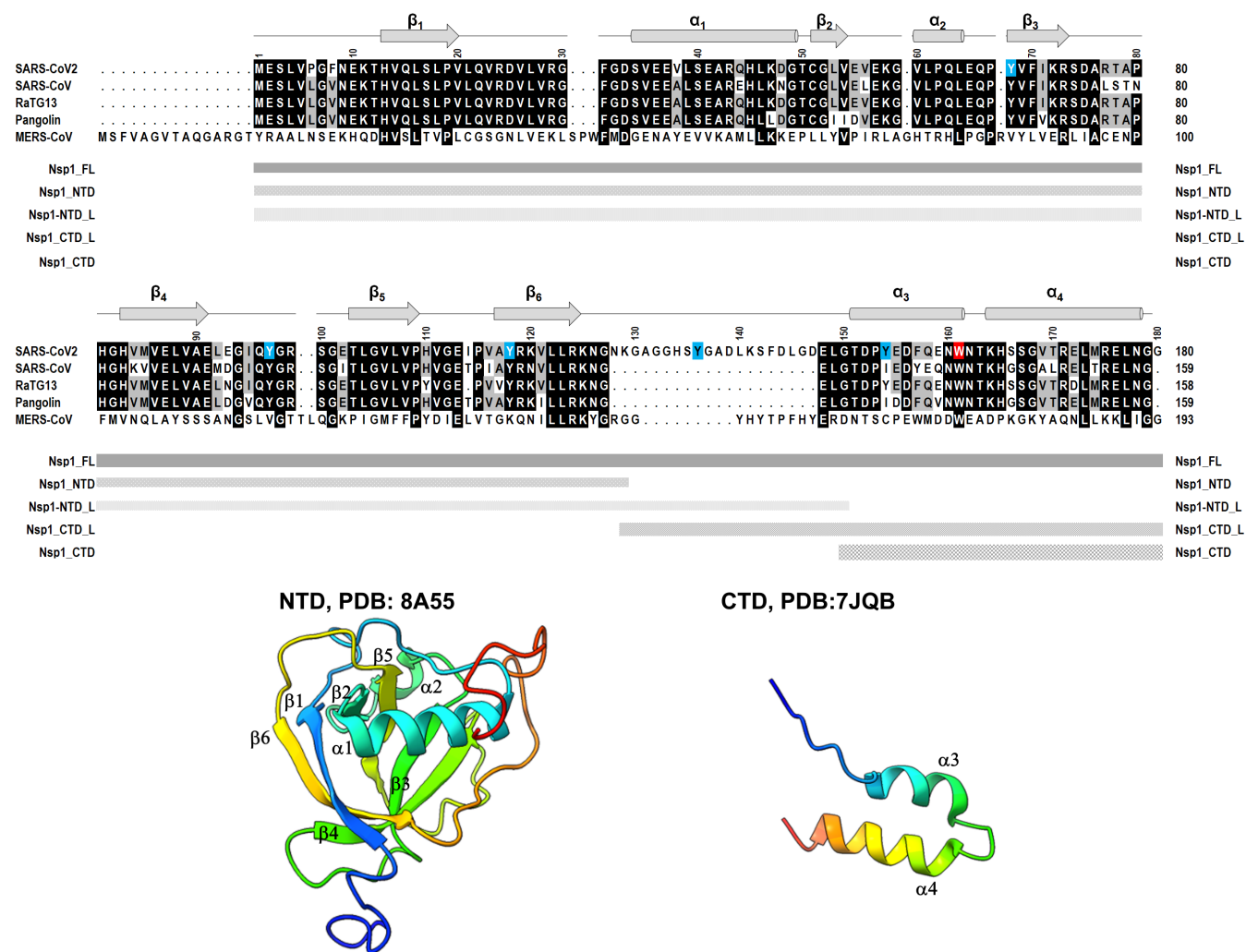


FIGURE 1 Nsp1 sequence alignment and its truncated forms. Sequence alignment of Nsp1 from genera *Sabercovirus* (SARS-CoV2; SARS-CoV; RaTG13; Pangolin) and *Merbecovirus* (MERS-CoV). The strictly conserved residues among all sequences excluding the MERS-CoV sequence are enclosed in black boxes, and the gray boxes denote conserved residues among three of the five sequences represented, all tyrosine residues are marked as blue, and the tryptophan is in red. The secondary structure of the Nsp1^{SARS-CoV2} is represented above the alignment, in which arrows represent β -strands and the cylinders represent the α -helices, based on the structures of the N-terminal domain obtained by X-ray crystallography (PDB:8A55), and of the C-terminal domain obtained from single particle Cryo-EM (PDB:7JQB). The cartoon representation of the N-terminal domain (PDB:8A55) and C-terminal domain (PDB:7JQB) is presented in the bottom panel. The residue range of the different constructs is presented below the sequence alignment. Nsp1_FL: SARS-CoV2 Nsp1 full-length; NTD: N-terminal domain, residues 1–129; NTD_L: N-terminal domain and loop, residues 1–150; CTD_L: Loop and C-terminal domain, residues 129–180; CTD: C-terminal domain, residues 150–180.

Hasoksuz et al. 2020). Nsp1 from SARS-CoV2 (Nsp1^{SARS-CoV2}) shares 84% sequence identity with Nsp1 from SARS-CoV, but only about 14% with its MERS-CoV (Middle East Respiratory Syndrome Coronavirus) counterpart (Min et al. 2020) (Figure 1).

Nsp1^{SARS-CoV2} protein is organized into two structural domains connected by a flexible linker: the N-terminal domain (NTD, residues 1–129) and the C-terminal domain (CTD, residues 150–180) (Figure 1). The structure of the full-length Nsp1^{SARS-CoV2} has been determined by NMR (Kaub et al. 2024; Wang et al. 2023), and the

NTD structure by X-ray crystallography (Borsatto et al. 2022; Clark et al. 2021; Ma et al. 2022; Semper et al. 2021; Zhao et al. 2021). The CTD structural architecture has been revealed by Cryo-Electron Microscopy (CryoEM) in complex with the 40S ribosomal subunit (Schubert et al. 2020; Thoms et al. 2020; Yuan et al. 2020).

The NTD of Nsp1^{SARS-CoV2} is a globular α/β domain, with a β -barrel arrangement composed of both anti-parallel and parallel β -strands, along with three helices (Borsatto et al. 2022; Clark et al. 2021; Kaub et al. 2024;

Ma et al. 2022; Semper et al. 2021; Wang et al. 2023; Zhao et al. 2021) (Figure 1). Structural data reveal a positively charged patch at the end of the β 6-strand, containing the conserved motif L₁₂₃RKXGXKG₁₃₀ (Figure 1), proposed to be important for RNA binding (Borsatto et al. 2022; Wang et al. 2023). Studies on the R124A/K125A variant of Nsp1 showed that this double variant abolishes RNA cleavage, due to a reduced positively charged region, thereby compromising RNA binding (Abaeva et al. 2023; Mendez et al. 2021; Salgueiro et al. 2024; Shehata and Parker 2023).

CryoEM studies reveal that the CTD folds into two helices (spanning amino acid residues 154–160 and 166–179) and inserts deeply within the mRNA entry channel of the ribosomal subunit (Schubert et al. 2020; Thoms et al. 2020; Yuan et al. 2020). Previous NMR, CD, and modeling studies highlighted the CTD's pronounced flexibility and its potential interactions with the NTD (Kumar et al. 2021; Salgueiro et al. 2024; Wang et al. 2023).

Nsp1's cellular functions so far reported include inhibiting type I interferon response at the translational level, suppressing host translation, inducing mRNA degradation, and blocking mRNA export (Burke et al. 2021; Gustin et al. 2009; Kaub et al. 2024; Nakagawa and Makino 2021; Schubert et al. 2020; Simeoni et al. 2021; Thoms et al. 2020; Yuan et al. 2020; Zhang et al. 2021; Zhao et al. 2021). According to previous studies, the CTD is crucial for Nsp1 ribosome co-localization and inhibit host protein translation by engaging into the 40S ribosomal subunit (Schubert et al. 2020; Thoms et al. 2020; Yuan et al. 2020). The linker between the NTD and CTD also contributes to protein translation inhibition (Banerjee et al. 2020; Shi et al. 2020; Vora et al. 2022). Conversely, NTD does not directly halt protein translation or ribosomal co-localization. Instead, it influences translation inhibition, potentially acting as a gatekeeper at the ribosome's mRNA entrance site (Zhao et al. 2021). The full-length Nsp1 and its CTD exhibit cytosolic localization, whereas the NTD alone displays both nuclear and cytosolic localization (Vora et al. 2022; Zhang et al. 2021).

Recently, we reported Nsp1 nuclease activity which targets both RNA and DNA substrates (Salgueiro et al. 2024). This activity is independent of the presence of ribosome and is modulated by metal ions like Ca²⁺, Mn²⁺, or Mg²⁺. Additionally, the R124A/K125A variant, with mutations located within the NTD, has lost its RNA cleavage capability while retaining its ability to cleave DNA (Salgueiro et al. 2024). However, the specific function of each domain remained unclear.

To gain a comprehensive understanding of the functions inherent to each domain, we have undertaken an

extensive biophysical and biochemical analysis using four truncated constructs of Nsp1^{SARS-CoV2}. Our findings highlight distinct roles for the different domains, namely the NTD is involved in regulating nucleic acid binding whereas the CTD serves as the catalytic domain for nucleolytic activity, which becomes inactive in membrane-like environments, such as detergent micelles and membrane fraction from *Escherichia coli*. These insights offer valuable information for drug targeting Nsp1^{SARS-CoV2}.

2 | RESULTS

2.1 | Production of Nsp1 truncated versions

To unveil the molecular mechanisms associated with the nucleolytic activity and gain insights into the role of each structural domain, we have generated four truncated versions of Nsp1^{SARS-CoV2} (Nsp1_FL): N-terminal domain (NTD, amino acids (a.a.) 1–129); N-terminal domain with the linker (NTD_L, a.a. 1–150); C-terminal domain with the linker (CTD_L, a.a. 129–180); and C-terminal domain (CTD, a.a. 150–180) (Figure 1).

All four truncated versions were successfully expressed; however, only three of them (NTD, NTD_L, and CTD_L) could be isolated from the soluble fraction and effectively purified (Figure 2). The NTD (0.9 ± 0.05) and NTD_L (1.1 ± 0.03) constructs eluted as monomers (Figure 2a–c), while the CTD_L construct eluted as a dimer (2.1 ± 0.1) (Figure 2d), as revealed by size-exclusion chromatography (SEC) analysis.

Notably, during the purification of NTD, it became evident that this truncated version was co-purified with nucleic acids (Figure 2b). To determine whether the co-purified nucleic acids were RNA or DNA, we incubated the sample with a commercial RNase or DNase and then analyzed it on an agarose gel. Our results demonstrated that the band was nearly degraded in the presence of RNase, while DNase had almost no effect (Figure 2e). This indicates that the NTD was purified mostly in the presence of RNA. Still, there is a small fraction that contains DNA.

During the production of the different Nsp1 variants, we observed that the CTD construct was exclusively expressed in the membrane fraction (Figure 2f). To determine if this specificity was unique to the CTD or if other constructs also showed membrane association, we analyzed the expression levels of the different constructs in both soluble and membrane fractions (Figure 2f). The results revealed that the full-length protein and the constructs NTD_L and CTD_L were present in both soluble

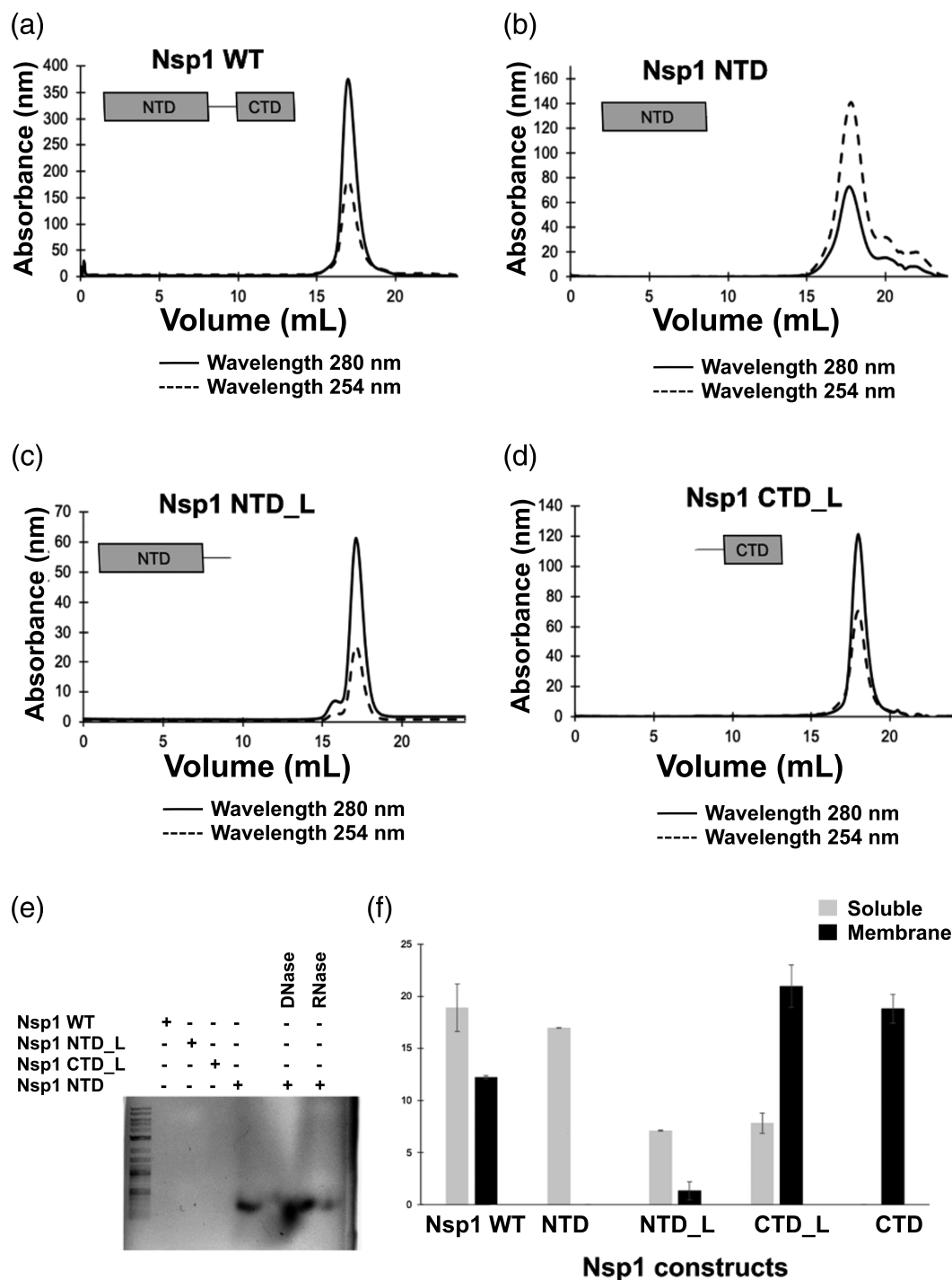


FIGURE 2 Expression analysis and purification of the Nsp1^{SARS-CoV2} and its truncated forms. Size exclusion chromatography (SEC) elution profile and quantification of the total protein amount of the full-length Nsp1^{SARS-CoV2} and the different truncated forms. (a) Full-length Nsp1^{SARS-CoV2}. (b) NTD: N-terminal domain, residues 1–129. (c) NTD_L: N-terminal domain and linker, residues 1–150. (d) CTD_L: C-terminal domain and linker, residues 150–180. In (a–d) absorbance at 280 nm is represented as solid lines and absorbance at 254 nm is indicated by dashed lines. (e) Agarose gel of the different truncated forms of the Nsp1^{SARS-CoV2} (Nsp1_FL, NTD, NTD_L, and CTD_L) and of the NTD truncated form incubated with DNase and RNase. (f) Quantification of the total amount of Nsp1_FL and the different truncated forms expressed in the soluble and membrane fractions, corresponding to three biological and three technical replicates. For panels (a–d), the conditions for the SEC were the following Superdex S-200 (Cytiva), using the buffer 20 mM HEPES pH 7.0, 250 mM NaCl, 10% (v/v) glycerol. The absorbance for the elution was measured at two wavelengths 280 and 254 nm.

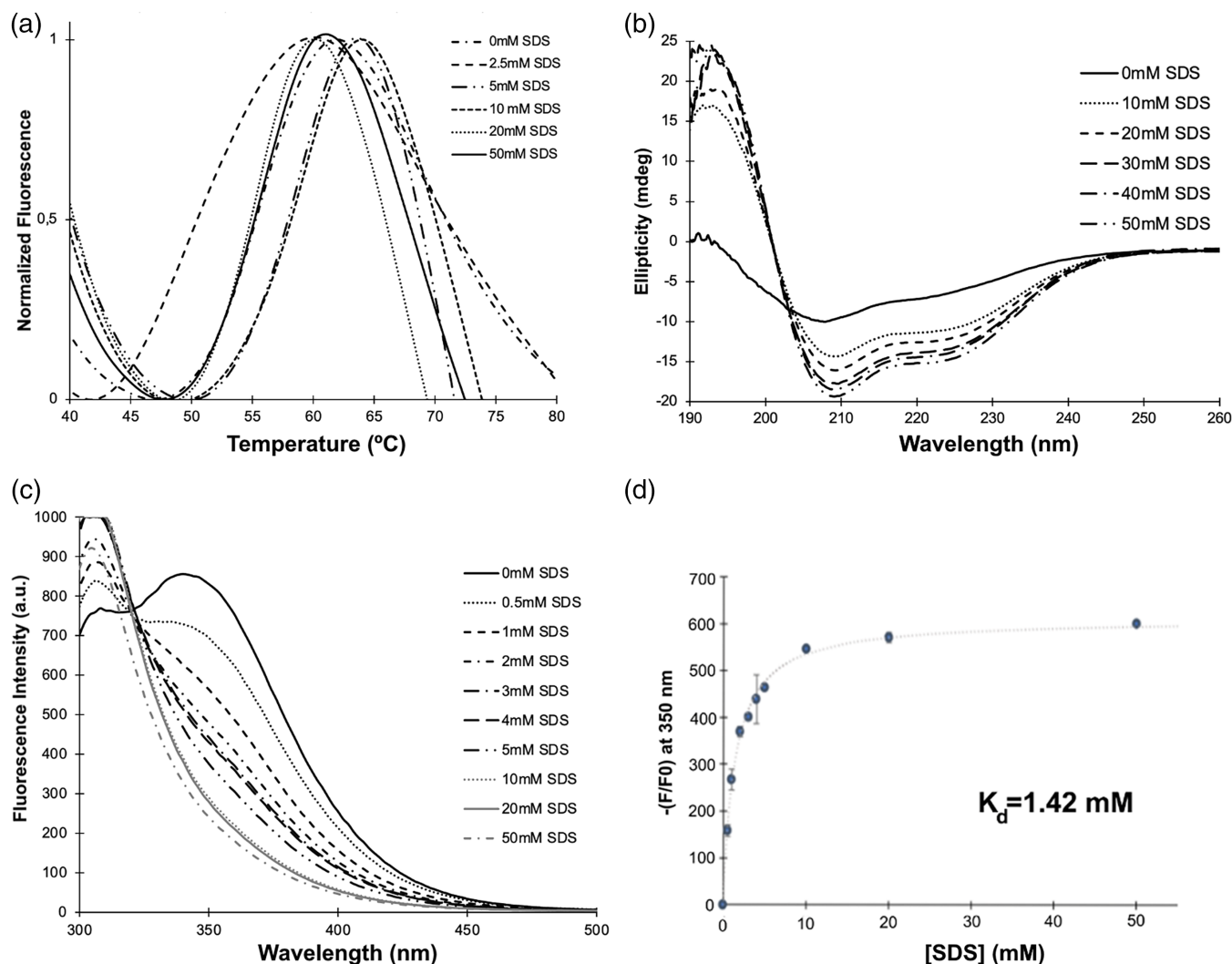


FIGURE 3 Biophysical characterization of the full-length Nsp1^{SARS-CoV2}. (a) Thermal shift assays with different SDS concentrations (2.5–50 mM). (b) Circular dichroism (CD) of Nsp1_{FL} in control conditions (black line) and with the addition of different SDS concentrations (10–50 mM, gray lines). (c) Tryptophan fluorescence spectra (excitation at 280 nm) in control conditions and in the presence of different concentrations of SDS (0.5–50 mM). (d) Tryptophan fluorescence at 350 nm plotted as a function of SDS concentration.

and membrane fractions, while the construct with only the NTD was exclusively expressed in the soluble fraction (Figure 2f). This suggests that the presence of the CTD or the linker (to a lesser extent) is associated with membrane localization.

2.2 | Probing Nsp1 affinity for hydrophobic environments

To further investigate the association of Nsp1, in particular the CTD, with hydrophobic regions, we assessed protein stability using conventional differential scanning fluorimetry (DSF) to determine the optimal SDS concentration for further studies. SDS is an anionic detergent commonly used to mimic membrane environment. It has

been previously employed in similar studies with other proteins, and its interaction with Nsp1^{SARS-CoV} has been reported (Kumar et al. 2021; Parker and Song 1992; Tulumello and Deber 2009). The results revealed that lower concentrations of SDS (2.5 mM) destabilize Nsp1_{FL}, resulting in a lower melting temperature (T_m) ($\Delta T_m = -6^\circ\text{C}$) compared to the protein in the absence of SDS (Figure 3a). At higher SDS concentrations (20–50 mM), the T_m was similar to that of the protein under control conditions, indicating relative stability under these conditions (Figure 3a). Surprisingly, SDS concentrations of 5–10 mM raised the T_m by 5°C (Figure 3a), possibly due to proximity to the critical micelle concentration (CMC) of SDS.

To complement these studies, we evaluated the secondary structural changes induced by SDS using circular

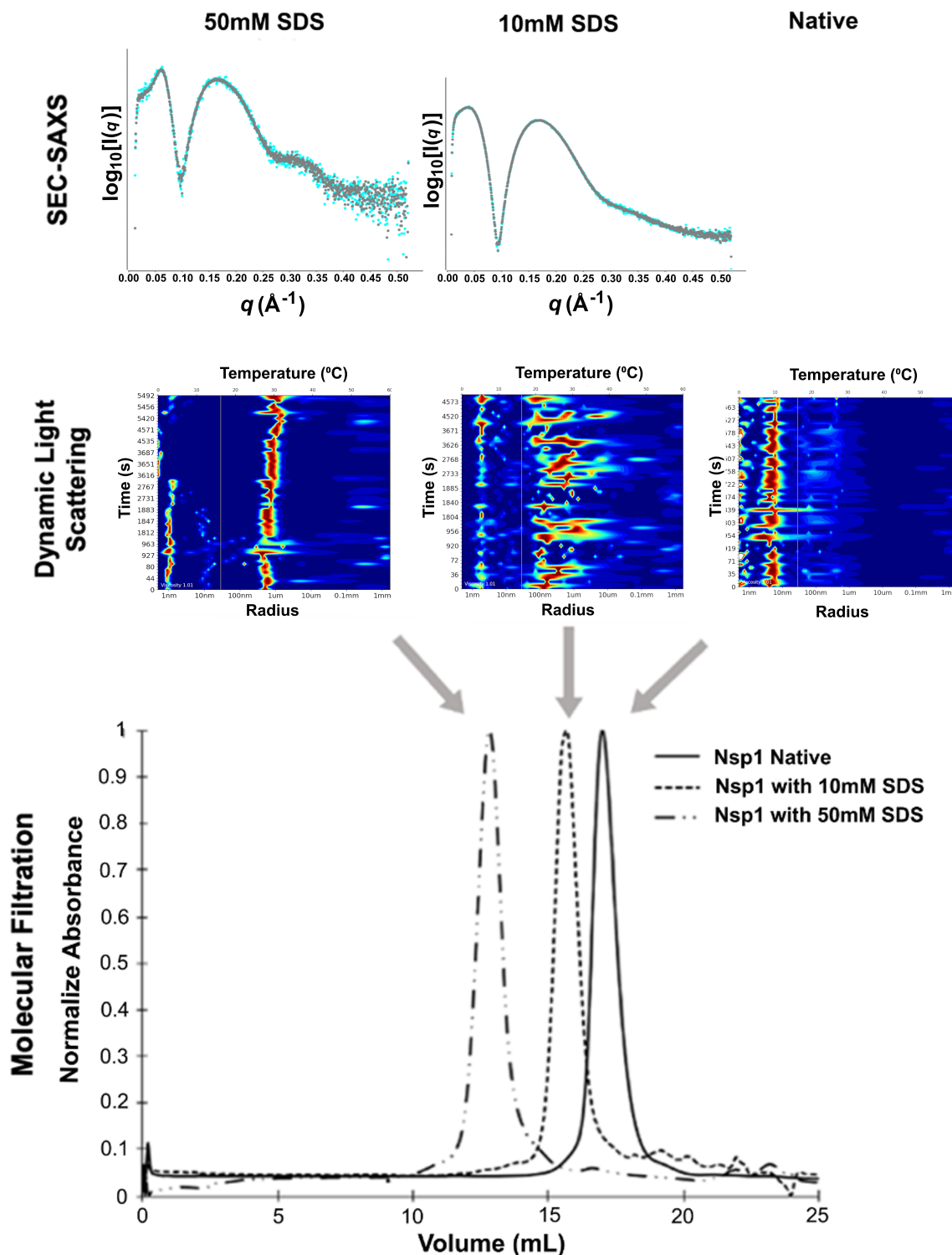


FIGURE 4 SDS effect on the full-length Nsp1^{SARS-CoV2}. (top) SEC-SAXS data of Nsp1_FL in the presence of two different concentrations of SDS (10 and 50 mM). (middle) Dynamic light scattering of full-length Nsp1_FL in native conditions and in the presence of different concentrations of SDS. (bottom) SEC of Nsp1_FL in native conditions (solid line), with 10 mM SDS (dashed line) and with 50 mM SDS (dashed-dotted line). The elution profiles were recorded by UV-VIS at 280 nm.

dichroism (CD) (Figure 3b). In native conditions, Nsp1_FL displayed α -helices in its secondary structure, as evidenced by small negative peaks at 208 and 222 nm,

consistent with previous data (Salgueiro et al. 2024). Upon adding SDS (10–50 mM), an increase in negative ellipticity at 208 and 222 nm, indicative of an α -helical

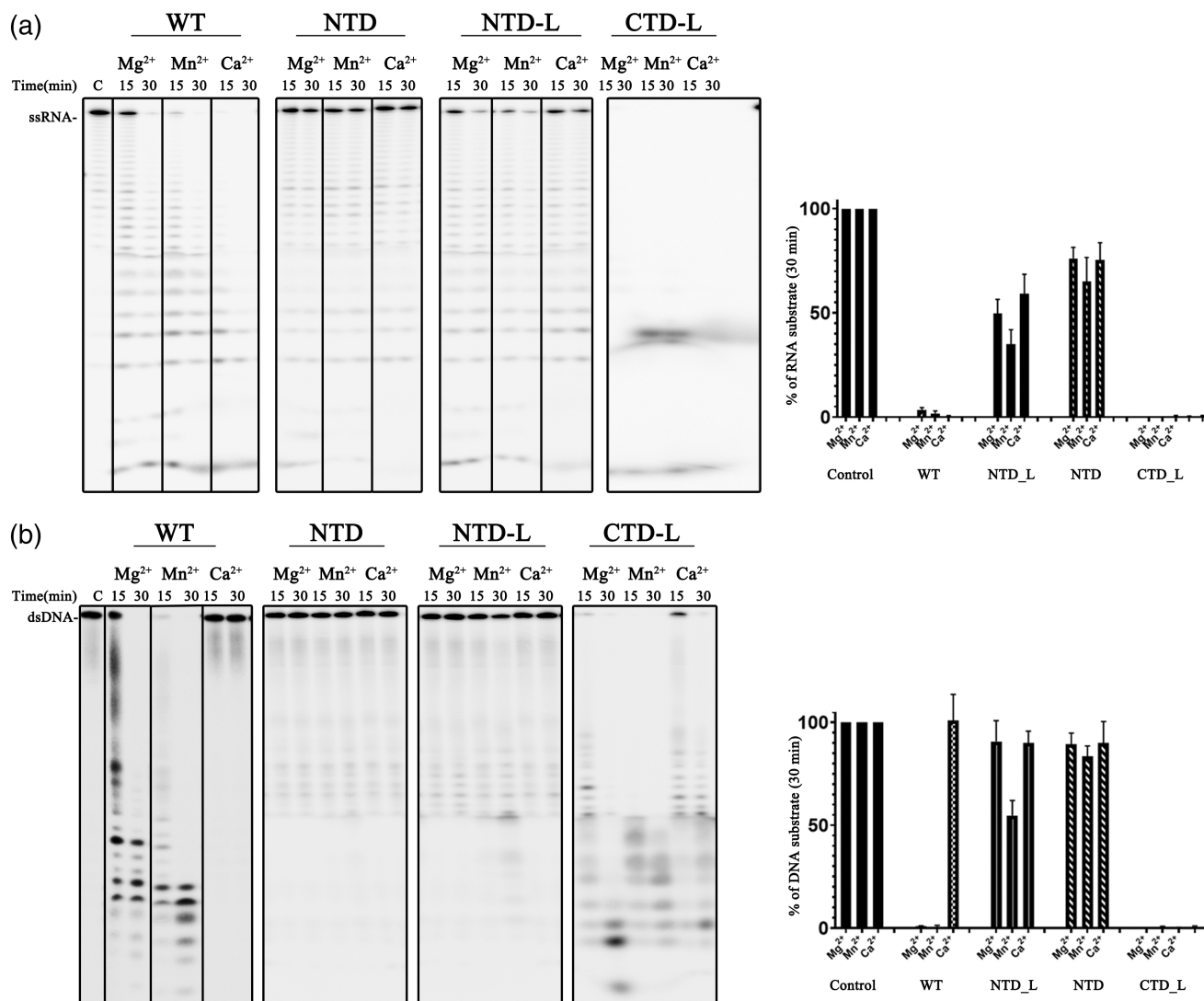


FIGURE 5 Nuclease activity of the full-length Nsp1^{SARS-CoV2} and its truncated forms. Endonuclease activity for the Nps1_FL and truncated forms, in the presence of the different metal ions. (a) ssRNA endonuclease activity. (b) dsDNA endonuclease activity. The quantification of the disappearance of the substrates is represented on the right side of both panels (ssRNA and dsDNA). For all the assays, a protein concentration of 5 μ M was used.

conformation, was observed. This shift in the CD spectra suggests a modification in the secondary structure, possibly involving the loop between the two domains adopting an α -helical conformation. Similar observations were made for the truncated version (131–180 a.a.) when subjected to the same SDS concentrations (Kumar et al. 2021).

Moreover, we took advantage of the single tryptophan (Trp161) present in the CTD (Figure 1), to monitor the decrease in tryptophan fluorescence, with increasing concentrations of SDS, until reaching a limit at SDS concentrations above the CMC (Figure 3c,d). This indicates that the tryptophan environment has changed, suggesting that the tryptophan is integrated into the SDS micelles, with a binding affinity of 1.42 mM (Figure 3d). This is

supported by our data showing that the presence of the CTD associates the protein with the membrane fraction (Figure 2f). Based on these findings, we selected two SDS concentrations for further studies: 10 mM (close to CMC) and 50 mM (above CMC).

Indeed, small-angle X-ray scattering (SAXS) studies revealed the presence of micelle-like spherical structures (Figure 4) with an estimated micelle core radius (R_c) of ~ 20 Å for each sample, and D_{max} values of 120 Å and 180 Å for the 10 mM and the 50 mM sample, respectively. Dynamic light scattering (DLS) analysis indicated higher homogeneity in the 50 mM SDS sample, likely due to the presence of micelles with a more uniform size compared to the 10 mM SDS sample (Figure 4). Furthermore, SEC analysis detected high-molecular-weight

oligomers in the presence of SDS, contrasting with the full-length protein profile (Figure 4). These findings highlight the critical role of the CTD in Nsp1's interaction with membrane-mimicking environments.

2.3 | Endonucleolytic activity

We have previously demonstrated that Nsp1_{FL}^{SARS-CoV2} displays nucleolytic activity towards RNA and DNA, independently of the presence of host ribosomes (Salgueiro et al. 2024). To further understand the role of each domain in the nuclease activity, we performed activity assays using the different Nsp1 constructs in the presence of Mg²⁺, Ca²⁺, or Mn²⁺ (Figure 5).

As already described, the NTD construct was co-purified with RNA (Figure 2b,e). Consequently, the nucleic acids were removed using a heparin purification step, prior to the activity assays. Upon conducting the degradation assays, we observed that the NTD is unable to degrade DNA but retained a residual activity against RNA, regardless of the metal ions used (Figure 5). The NTD_L version (Figure 5a) displayed a compromised RNA degradation ability when compared to the full-length protein and its DNA cleavage ability was almost abolished, showing only residual activity on DNA substrates in the presence of Mn²⁺ (Figure 5b). In contrast, CTD_L degraded both RNA and DNA more efficiently than the wild type in all the tested conditions (Figure 5). The substrates were rapidly degraded after 15 min of incubation with CTD_L (Figure 5). Interestingly, the CTD_L truncated version demonstrated DNA degradation ability in the presence of Ca²⁺, contrarily to the full-length protein. This truncated version also retained its ability to cleave RNA in the absence of divalent metals but was unable to degrade DNA, indicating a requirement for divalent metals for DNA degradation (Figure S1a, Supporting Information). To further confirm this observation, we tested the activity of Nsp1_{FL} and CTD_L on RNA in the presence of EDTA, a metal ion chelator (Figure S1b). The RNA nuclease activity for the full-length protein seems to be slightly reduced in the presence of EDTA considering that after 60 min of incubation we are still able to observe substrate (Figure S1b), while 30 min of incubation in the presence of metal ions is enough to completely degrade the full length substrate (Figure 5a). This observation supports our previous results that the protein activity is mediated by the presence of metals (Salgueiro et al. 2024), whereas the CTD_L truncated version retains its RNA nuclease activity in the absence of divalent metals (Figure S1).

As already mentioned, SDS forms micelles in solution, mimicking the environment of biological

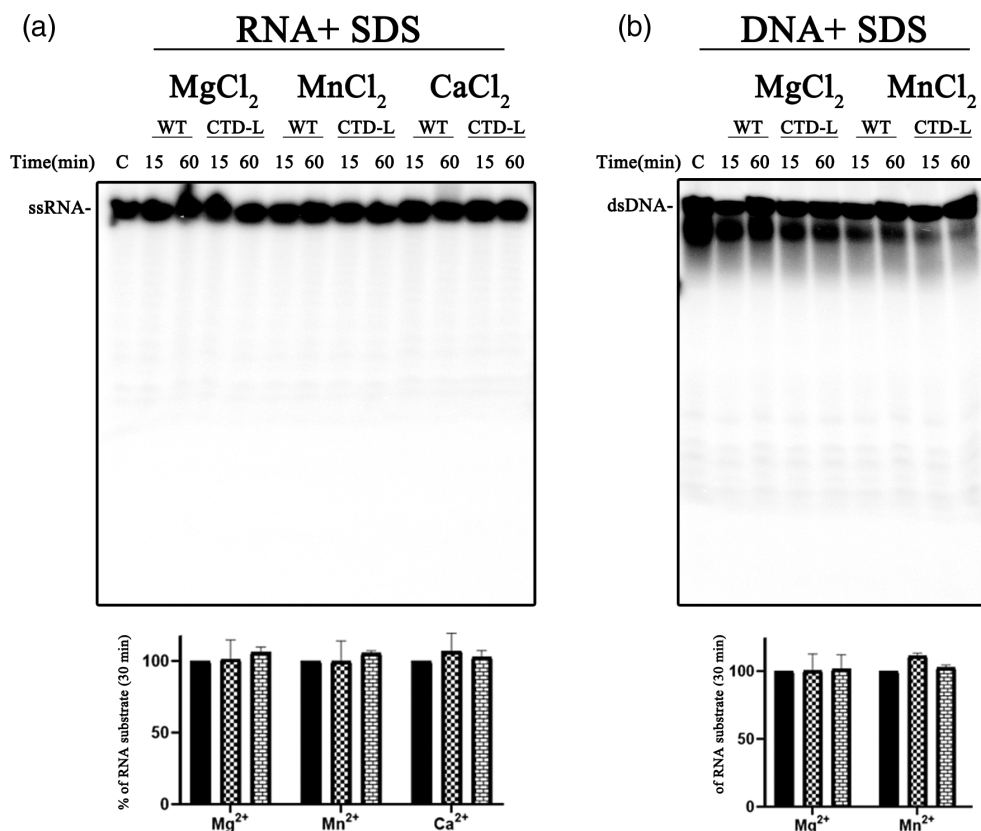
membranes (Tulumello and Deber 2009). To investigate the impact of SDS on the nucleolytic activity of Nsp1_{FL}, specifically in the CTD domain, we conducted activity assays in the presence of 10 mM of this detergent. At this SDS concentration, Nsp1_{FL} remains relatively stable with some modifications in its secondary structure (Figure 3a). However, our findings revealed that the protein loses its ability to degrade both RNA and DNA in the presence of SDS, under all conditions tested (Figure 6).

These findings underscore the CTD and NTD's critical roles in Nsp1's function. We suggest that the CTD serves as the catalytic domain and is responsible for the protein's nucleolytic activity, and when this domain is integrated into membrane-like environments, the protein becomes inactive. Additionally, the NTD appears to be responsible for binding RNA, as this domain was co-purified with RNA, probably because it had lost its ability to efficiently cleave RNA. Moreover, the NTD may act as a regulator of DNA cleavage by influencing the requirement for metal ions.

2.4 | Formation of the tyrosyl radical in DNA nuclease activity

The Nsp1_{FL} presents RNA/DNA nuclease activity which is mediated by the presence of metals (Figure 5) (Salgueiro et al. 2024). Structural data reveal a positively charged patch at the end of the β6-strand, containing the conserved motif (L₁₂₃RKXGXKG₁₃₀) (Figure 1), which is proposed to be important for RNA binding (Borsatto et al. 2022; Wang et al. 2023). Nsp1^{SARS-CoV2} does not share any sequence or structural homology with other nucleases (Yang 2011). However, it contains several charged residues (lysines, arginines, glutamates, aspartates, and tyrosines) that could be involved in the reaction mechanism, as in other nucleases (Yang 2011). Tyrosine residues are known to be involved in the reaction mechanism of nucleases, such as DNases, by forming tyrosyl radical species (Tyr-O•) due to the deprotonation of the hydroxyl group (Mundle et al. 2004). This radical performs a nucleophilic function, by binding to the phosphate group of the DNA chain and promoting its cleavage (Mundle et al. 2004; Yang 2011). The formation of this tyrosyl radical can be monitored through spectroscopic analysis, namely UV-visible (UV-VIS), by observing the appearance of a characteristic band around 410 nm (Tomter et al. 2011). To evaluate the formation of the tyrosyl radical during the nuclease step of the Nsp1^{SARS-CoV2} reaction mechanism, UV-VIS spectroscopy was performed on our different constructs Nsp1_{FL}, NTD, NTD_L, and CTD_L. Nsp1^{SARS-CoV2} contains five tyrosine residues (Y): Y68,

FIGURE 6 SDS effect on the endonuclease activity of Nsp1_FL and CTD_L truncated forms. Endonuclease activity for the Nps1_FL and CTD_L forms in the presence of the different metal ions and 10 mM of SDS. (a) ssRNA endonuclease activity. (b) dsDNA endonuclease activity. The quantification of the disappearance of the substrates is represented at the bottom of each panel. For all the assays, a protein concentration of 5 μ M was used.



Y97, Y118, present in the NTD, Y136 in the linker between the two domains and Y154 in the CTD (Figures 1 and 7). The different constructs were pre-incubated with Mn^{2+} , followed by adding dsDNA to the assay (Figure 7). Our results demonstrated the formation of the tyrosyl radical in the Nsp1_FL as demonstrated by a characteristic UV-VIS band at 410 nm (Figure 7a) immediately after the addition of dsDNA. In contrast, no band indicative of a tyrosyl radical was observed for the NTD construct (Figure 7b), consistent with its lack of nucleolytic activity (Figure 5). Our data suggest that Y68, Y97, and Y118 are not involved in the nucleophilic attack on dsDNA due to the lack of tyrosyl signal in the UV-VIS spectra (Figure 5). On the other hand, the NTD_L show the tyrosyl formation during the nuclease activity (Figure 7c), suggesting that Y136 could be involved in the nucleophilic attack. Nevertheless, our data from the CTD_L construct which includes both Y136 and Y154, showed no evidence of tyrosyl radical formation (Figure 7d). The absence of a UV-VIS signal in this construct could be caused by the fact it lacks the nucleotide binding site which is located on the NTD and thus is not possible to detect a stable tyrosyl radical. However, it is interesting to note that the CTD_L showed a higher activity compared with Nsp1_FL. In order to demonstrate the role of Y136 residue in the nucleotide activity, we generated the Y136F variant of Nsp1_FL, and tested its nucleotide activity. Our results show that while this variant is

unable to cleave DNA, it still retains the RNA nuclease activity (Figure 8).

3 | DISCUSSION

The Nsp1^{SARS-CoV2} protein is unique among α and β coronaviruses. It is structurally composed of two domains: the N-terminal domain (NTD), the C-terminal domain (CTD), and a loop connecting them. This protein is highly conserved across SARS-CoV2 strains, with approximately 94% of sequences showing no amino acid mutations. Among the few mutations observed in the emerging variants, the most frequent ones occur in the NTD, including R24C, E37D, E87D, S100N, and H110Y (Ghaleh et al. 2023; Min et al. 2020). However, only the residues E37D and E87D are close to the proposed nucleotide binding site that includes the conserved motif (L₁₂₃RKXGXKG₁₃₀). Still, both mutations are substitutions of a glutamate for an aspartate which does not yield any electrostatic potential change near the binding pocket. The other residues are not close to the proposed nucleotide binding site and thus are probably not involved in the nuclease reaction mechanism motif.

Given the critical role of Nsp1 and its highly conserved nature, it represents a promising target for therapeutic intervention. Thus, to effectively exploit this protein for therapeutic purposes, it is essential to gain a

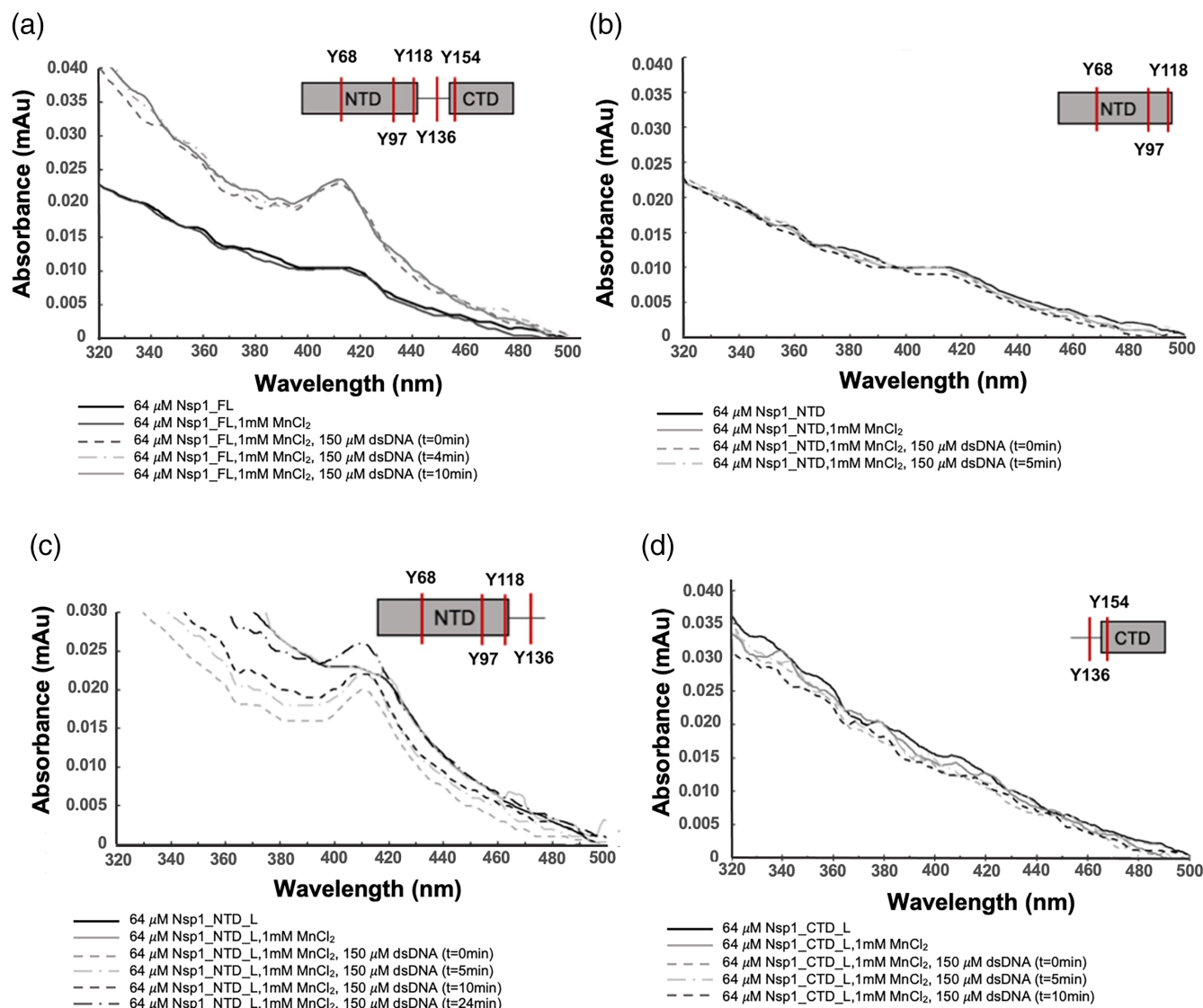


FIGURE 7 UV-VIS spectra of the tyrosyl radical formation during the Nsp1^{SARS-CoV2} DNA nuclease activity. (a) Nsp1_FL; (b) NTD; (c) NTD_L; (d) CTD_L. For all panels, the black line corresponds to the protein alone, the dark gray line corresponds to the protein pre-incubated with 1 mM MnCl₂, and the light gray lines correspond to the addition of dsDNA to the previous mixture at different time points (0, 5, and 10 min). For panel (c) (NTD_L) an extra time point was measured at 24 min.

more comprehensive understanding of its functional mechanisms.

Various functions have been attributed to Nsp1^{SARS-CoV2}, highlighting its multifunctional nature (Burke et al. 2021; Salgueiro et al. 2024; Schubert et al. 2020; Simeoni et al. 2021; Thoms et al. 2020; Yuan et al. 2020; Zhang et al. 2021). Recently, we demonstrated that Nsp1_FL^{SARS-CoV2} exhibits nucleolytic activity independent of ribosomes and mediated by divalent metals (Salgueiro et al. 2024). In this study, we aimed to dissect the contributions of each structural domain regarding the nuclease activity. For this purpose, we generated four truncated versions of Nsp1^{SARS-CoV2}: NTD (a.a. 1–129), NTD_L (a.a. 1–150), CTD_L (a.a. 29–180), and CTD (a.a. 150–180) (Figure 1).

Our findings revealed distinct roles for the NTD and CTD regarding the nucleolytic activity of Nsp1^{SARS-CoV2}. The NTD appears to play a regulatory role, possibly in binding nucleic acids, as this domain binds to RNA without degrading it. Accordingly, both the NTD and the NTD with the linker (NTD_L) exhibited significantly compromised RNA degrading activity when compared with the full-length protein (Figure 5). Furthermore, we showed that the NTD completely lost its ability to degrade DNA. In contrast, the C-terminal domain with the linker (CTD_L) displayed enhanced activity and appears to be the primary domain responsible for nucleic acid cleavage (Figure 5). CTD_L could cleave RNA independently of metal ions, while DNA cleavage required the presence of metals (Figures 5 and S1). This suggests

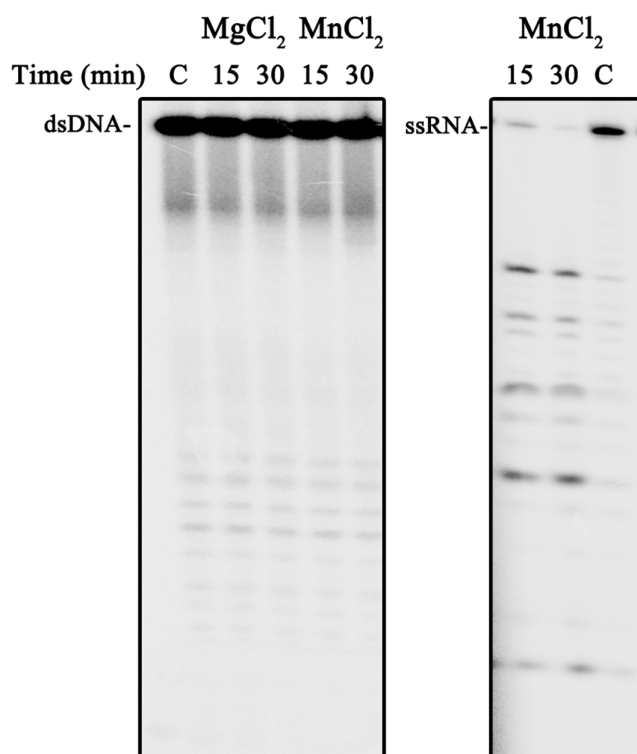


FIGURE 8 Nuclease activity of the Y136F full-length Nsp1^{SARS-CoV2} variant. dsDNA endonuclease activity for the Y136F Nps1_FL variant was tested in the presence of the magnesium and manganese (left) and the ssRNA endonuclease activity was tested in the presence of manganese. For all the assays, a protein concentration of 5 μ M was used.

that the NTD also influences substrate cleavage through metal ion interactions. These findings underscore the crucial interplay between the NTD and CTD, mediated by the linker loop, and the presence of metals in regulating Nsp1's nuclease activity.

Indeed, the NMR structure of Nsp1^{SARS-CoV2} (Wang et al. 2023) depicts an interaction between the CTD and NTD, forming a globular structure, with the N-terminal region up to residue 11 interacting with the nucleotide binding pocket. Moreover, it has also been reported that the absence of the first 12 N-terminal amino acid residues is associated with a lower viral load and reduced efficiency in promoting Nsp1 evasion during viral translation (Sosnowski et al. 2022; Tardivat et al. 2023; Wathelet et al. 2007). This supports our observations that the interplay between the NTD and CTD is essential for proper Nsp1 function.

Previous studies have also suggested that the endonuclease activity of Nsp1 was located in the NTD, supported by findings from studies on the Nsp1 variants R99A and R124A/K125A, both of which do not exhibit nuclease activity (Abaeva et al. 2023; Tardivat et al. 2023). The residues R124 and K125 are part of the conserved amino acid sequence LR₁₂₄K₁₂₅XGxK₁₂₉G (Figure 1a), which

plays an important role in RNA binding (Almeida et al. 2007; Libich and Baudin 2023; Lokugamage et al. 2012; Tanaka et al. 2012; Terada et al. 2017; Wang et al. 2023). Therefore, the loss of nucleolytic activity in the variants R99A and R124A/K125A is likely due to changes in the electrostatic potential surface of the nucleotide-binding pocket, impairing substrate binding.

Structural comparisons of available Nsp1 structural models (Schubert et al. 2020; Wang et al. 2023) reveal distinct conformations of the C-terminal domain, consistent with its known flexibility (Salgueiro et al. 2024). Our data indicate that the CTD is the domain responsible for cleavage.

Considering the role of Nsp1^{SARS-CoV2} as a nuclease, we have performed sequence and structural comparisons with known nucleases in order to identify possible similarities which could reveal potential residues or mechanisms responsible for the nucleolytic activity. However, they did not yield significant results. Catalytic residues in nucleases typically include histidine, aspartate/glutamate, and tyrosine. Histidine and aspartate/glutamate residues often participate in the catalytic mechanism by coordinating metal ions. Tyrosine plays a crucial role in the nucleophile attack, by forming a phosphotyrosyl radical during nucleotide-protein interaction (Yang 2011). Additionally, lysine and arginine residues are commonly involved in nucleotide interactions (Yang 2011). Analysis of the amino acid sequence of Nsp1^{SARS-CoV2} compared with other Nsp1 proteins reveals a high conservation of these catalytic residues (Figure 1). Within the nucleotide binding pocket of the NTD, three histidine (H13, H81, and H83) are positioned near residues R124–K125, potentially critical for the protein's activity. Furthermore, we observed the presence of a tyrosyl radical during a nuclease reaction, identified by a characteristic band at 410 nm detected by UV–VIS spectroscopy (Figure 7).

Data from the different constructs tested in this work suggest that the tyrosine residue Y136 could be involved in the reaction mechanism. Although this residue is present in the CTD_L construct, no tyrosyl was detected in this construct under the conditions tested. This absence may be attributed to the fact that the reaction mechanism for nucleases, in which a tyrosyl radical is formed, requires a first step that is the nucleotide binding followed by the nucleophilic attack by the tyrosine residue (Deweese and Osheroff 2009; Yang 2011). Our data, indicate that the NTD contains the nucleotide binding site LR₁₂₄K₁₂₅XGxK₁₂₉G, and the tyrosine Y136 would potentially perform the nucleophilic attack. In fact our results on the Y136F variant of Nsp1_FL show that this variant lacks the DNA nuclease activity, while retaining RNA nuclease activity. This result is particularly interesting because our previous results demonstrated that the R124AK125A variant of Nsp1_FL exhibits the opposite

behavior, it does not degrade RNA but retains the ability to cleave the DNA (Salgueiro et al. 2024). This suggests that the mechanisms by which the protein interacts with both nucleotides differ.

Furthermore, the reaction mechanism of CTD_L may differ from that of the NTD_L or Nsp1_FL, potentially involving other residues. Notably, CTD_L presents higher activity than the full-length protein, which masks the possibility to detect a stable tyrosyl. Nevertheless, we cannot exclude that Y154 could also play a role in the nuclease activity. While we currently lack specific information about the residues involved in CTD_L nuclease activity, previous studies have highlighted the importance of residues K164 and H165 in ribosome binding (Tardivat et al. 2023). Additionally, arginine residues R171 and R176 may contribute to a positively charged electrostatic patch crucial for nucleotide interaction.

Comparing the various Nsp1 sequences (Figure 1), the residue Y136 is localized in the linker region between both domains NTD and CTD, and Y154 is located at the CTD. Interestingly these residues are not conserved among the Nsp1 protein sequences in the different coronaviruses (Figure 1). But MERS-CoV Nsp1 contains three tyrosine residues (Y153, Y155, Y160) in the NTD-CTD linker region, the RaTG13 Nsp1 contain a conserved tyrosine residue in the same position as Y154, and SARS-CoV Nsp1 contain also a tyrosine residue Y137 close by (Figure 1).

Previous studies have reported the interaction of the CTD of Nsp1 with the ribosome to inhibit host protein synthesis, facilitating the cleavage of host mRNAs (Schubert et al. 2020; Shehata and Parker 2023; Tidu et al. 2021). In our study, we demonstrate that the CTD alone localizes the protein to the membrane fraction (Figure 2). Furthermore, mimicking membrane environments *in vitro* by adding SDS abolished the ability of both full-length and CTD_L proteins to degrade RNA and DNA (Figures 3, 4, and 6). Moreover, we observed that the fluorescence of tryptophan in Nsp1_FL decreases in the presence of SDS. This suggests that the tryptophan may be inserted into the SDS micelles. This observation is consistent with a previous study that analyzed the CTD (a.a. 131–180) of Nsp1^{SARS-CoV2} in the presence of SDS, TFE and liposomes. In that study, a similar decrease in the tryptophan fluorescence was observed when these components were present, suggesting a conformational change of tryptophan to a more hydrophobic environment (Kumar et al. 2021). However, since no endonucleolytic activity was detected in the presence of SDS, we can propose that under the presence of SDS the CTD is in fact inserted into the SDS micelles.

Nevertheless, it remains unclear whether Nsp1s association with the cell membrane or detergent micelles is related to a cellular function, it does raise intriguing

possibilities. This is especially noteworthy considering previous work where Nsp1^{SARS-CoV2} was observed to associate with the nucleoporin Nup93, displacing it from the nuclear pore complex (Gomez et al. 2019; Sajidah et al. 2021). Moreover, prior studies suggest that Nsp3, Nsp4, and Nsp6 are potential pore components in double membrane vesicles, with Nsp3 and Nsp4 sufficient to form a pore spanning the vesicles (Wolff et al. 2020; Zimmermann et al. 2023). It remains an open question whether other viral proteins can be associated with these vesicles. Based on the previous reports and our observations it is tempting to suggest that Nsp1 could be involved in this process; however, this remains to be confirmed.

In summary, our comprehensive investigation of Nsp1^{SARS-CoV2} has provided valuable insights into the functional role of each structural domain. Specifically, the NTD appears to play a regulatory role, potentially involved in binding nucleic acids and influencing substrate cleavage through metal ion interactions. Furthermore, our data indicate that the CTD holds the catalytic residues and is responsible for the nucleolytic activity. Additionally, we have demonstrated the ability of Nsp1^{SARS-CoV2} to interact with hydrophobic environments, potentially associating with cellular membranes under *in vivo* conditions. These findings not only advance our understanding of the role of Nsp1^{SARS-CoV2} but also provide a solid foundation for future studies exploring its roles in viral infection and host interactions. Understanding these mechanisms is crucial for developing therapeutic strategies against SARS-CoV2 and other related coronaviruses.

4 | MATERIALS AND METHODS

4.1 | Protein expression and purification

The genes encoding for the different Nsp1^{SARS-CoV2} constructs and the Y136F Nsp1_FL variant were cloned in the pET28a(+)TEV vector from the pET28a(+)-Nsp1 plasmid ending for the previously used Nsp1^{SARS-CoV2} full-length protein (Salgueiro et al. 2024). Codons were optimized for *Escherichia coli* expression, and the plasmids were obtained from GenScript (Rijswijk, The Netherlands).

The protocol for protein expression and purification is similar to that previously described (Salgueiro et al. 2024). After purifying the different Nsp1 constructs, the oligomerization state was determined at room temperature with a Superdex 200 column (10/300 GL, Cytiva) using a 20 mM HEPES pH 7.0, 250 mM NaCl, 10% (v/v) glycerol buffer and different SDS concentrations. The column was calibrated using standard proteins ranging from 14 to 660 kDa, the distribution coefficient (K_{av}) for each

protein standard was plotted versus its log MW for corresponding standards, blue dextran was used to determine V_0 (Cytiva). The molecular weight determination for each Nsp1 construct was then carried out taken in consideration the calibration curve with the standards. The elution of the proteins was monitored by UV absorption at 280 and 254 nm.

The small-scale bacterial growths (50 mL) for the different Nsp1 constructs were performed as previously described (Salgueiro et al. 2024). Cells were lysed using a French press (900 psi $\times$ 3 cycles) and the buffer 20 mM HEPES pH 7.0, 10% (v/v) glycerol, 300 mM NaCl, 50 mM L-Arginine, 50 mM L-glutamic acid, 10 mM $MgCl_2$, 1 μ g/mL DNase I, and 0.1 mg/mL Lysozyme. The extracts were centrifuged at 48,384g for 60 min at 10°C (Beckman Avanti J-26). The pellet was resuspended in 20 mM HEPES pH 7.0, 10% (v/v) glycerol, 300 mM NaCl, 50 mM L-Arginine, 50 mM L-glutamic acid, 0.05% Triton X-100 and centrifuged at 4500g for 10 min at 4°C (Eppendorf centrifuge 5415R). The protein quantification of the soluble fractions and the membrane fractions was performed using the BCA Protein Assay (Pierce) (Smith et al. 1985). A 15% SDS-PAGE was performed in which a total of 10 μ g of protein was loaded for the different samples. The Western-Blot was performed in the wet-system (BioRad), using as transfer buffer 48 mM Tris-base, 39 mM glycine pH 9.2, 20% (v/v) methanol and the blotting conditions at 4°C were 30 V overnight, followed by 100 V for 30 min. The analysis consists of three biological replicates and three technical replicates. The bands from the Nsp1 protein constructs were detected using Anti-polyHistidine antibody (Sigma) and imaged with the iBright imaging system. The band quantification was performed using the FIJI software (ImageJ 1.54f) (Schindelin et al. 2012).

4.2 | Biophysical characterization

Fluorescence-based thermal shift assays were carried out on an iCycle iQ5 Real Time PCR Detection System (Bio-Rad) as previously described (Salgueiro et al. 2024). Each reaction mixture of 22 μ L, containing 7.5 μ g of Nsp1 purified protein, 25 $\times$ SYPRO orange dye (Molecular Probes) and different concentrations of SDS, was subjected to thermal denaturation on a 96-well plate (low profile plate, Bio-Rad) from 20 to 90°C with an increment of 0.5°C/min, and the resulting fluorescence data were fitted to a standard four-parameter Boltzmann sigmoidal equation.

Circular dichroism (CD) spectra were measured using a JASCO J-850 CD spectrometer connected to a Peltier temperature controller, as previously described (Salgueiro et al. 2024). All the assays were performed in 20 mM sodium phosphate buffer pH 6.5, with a protein

concentration of 0.1 mg/mL. The protein samples were incubated with different concentrations of SDS. The percentages of the different secondary structure elements were calculated using single spectra analysis in Beta Structure Selection—BeStSel—webserver (Micsonai et al. 2022a; Micsonai et al. 2022b).

We monitored the intrinsic tryptophan fluorescence intensity in Nsp1_FL using a Cary Eclipse Fluorescence Spectrophotometer. A total of 5 μ M protein sample in 20 mM sodium phosphate buffer (pH 7.0) was prepared with increasing SDS concentrations (0.5–50 mM). Emission spectra were recorded from 300 to 500 nm with a slit width of 10 nm and a scan speed of 1200 nm/min. The excitation wavelength was fixed to 280 nm and the temperature was kept constant at 25°C by using an external thermostatic water circulator.

Dynamic light scattering (DLS) measurements of the different conditions of Nsp1_FL protein was analyzed on a Spectroscatter 201 (XtalConcepts). Prior to measurements, the protein was centrifuged for 30 min at 13,000 rpm. A series of measurements (50–70) were collected with a sampling time of 45 s by applying a scattered red-light laser ($\lambda = 659$ nm) at a fixed angle of 142° and a temperature of 16.2°C.

4.3 | SAXS data collection, processing, and ab initio modeling

SAXS data were collected at the ESRF BioSAXS beamline BM29 (Tully et al. 2023) from Nsp1_FL native and with 10 and 50 mM SDS. An inline HPLC system (Shimadzu) was used (Tully et al. 2021) coupled directly to the BM29 via a quartz capillary in vacuum. The protein concentration used was 7.5 mg/mL in the buffer 20 mM HEPES pH 7.0, 250 mM NaCl, 10% (v/v) glycerol, 50 mM L-arginine, and 50 mM glutamic acid with either 10 or 50 mM SDS. A Superdex 200 10/300 GL (GE Healthcare) column was used and equilibrated with 10 column volumes of buffer at room temperature. SAXS data were collected using X-rays of wavelength of 0.9919 Å (12.5 keV) and a sample-to-detector distance of 2.81 m corresponding to a q -range of 0.007–0.5 Å^{−1}, where q is the magnitude of the scattering vector given by $q = 4\pi/\lambda \sin(\theta)$, with 2θ the scattering angle. A total of 1200 frames (at 2 s/frame) were collected from each protein sample.

Each individual frame was integrated by the beamline-integrated automated processing pipeline using FreeSAS (Kieffer et al. 2022) before buffer subtraction, and primary data processing was carried out manually using Scatter IV (Tully et al. 2021). The SEC-SAXS could not completely separate the protein from the aggregates, leading to overlapping peaks. To determine the number of distinct scatterers and extract the individual

components, the program BioXTAS RAW, which encompasses a single value decomposition (SVD) with evolving factor analysis (EFA) tool was used (Meisburger et al. 2016; Tully et al. 2021).

4.4 | Electrophoretic mobility shift assay

An agarose gel (0.8% (w/v)) was prepared using 0.5X TAE buffer and supplemented with 2.5 μ L of Xpert Green DNA Stain (Grisp). For each well, 10 μ L (~2 mg/mL) of different constructs of Nsp1^{SARS-CoV2}: NTD; NTD_L, and CTD_L and the Y136F Nsp1_FL variant was loaded. The gel was run at room temperature in 0.5X TAE buffer at 10 V for 40 min and was visualized using the Gel DocTM EZ system (BioRad).

4.5 | UV/VIS spectroscopic assay

UV-visible spectra of purified Nsp1_FL protein and different constructs were measured on a UV-1800 Shimadzu UV spectrophotometer in the 200–500 nm wavelength range using agitation at room temperature (25°C). Experiments were performed under aerobic conditions and using a 10 mm quartz cuvette. The protein (64 μ M) was previously incubated with 1 mM MnCl₂ in the buffer 20 mM HEPES pH 7, 250 mM NaCl, 10% (v/v) glycerol, 20 mM L-glutamic acid, and 20 mM L-arginine followed by the addition of 150 μ M of DNA substrate hybridized to the complementary oligonucleotide (5'-GAACAGCTCACTGATTTACCCAAA TGGGAAAAAGG-3').

4.6 | RNA and DNA 5'-end labelling

The following synthetic oligonucleotide (StabVida, Portugal) was used as substrate in the activity assays: 30-mer RNA (5'-CCCACACCAACCACUAAAAAAAAA AAAA-3'). Each oligonucleotide was labeled at its 5' end with [³²P]- γ -ATP and T4 Polynucleotide Kinase (Ambion) in a standard reaction. MicroSpin G-50 columns (Cytiva) were used to remove the excess of [³²P]- γ -ATP. In order to fold them into their secondary structures, the substrates were resuspended in 10 mM of Tris-HCl pH 8.0 and incubated 10 min at 80°C followed by 30 min at 37°C. The 31-mer DNA substrate was also hybridized to the complementary non-labeled 31-mer oligonucleotide (5'-CGCACGTTGCCAGCTGCGCTGCGAT CATGAC-3') added in excess (molar ratio 1:10) in order to obtain a perfect duplex. The hybridization was performed by incubation for 10 min at 80°C followed by a final step of 30 min at 37°C.

4.7 | In vitro activity assays

The activity assays were performed in the presence of the activity buffer (20 mM HEPES pH 7.4 and 10 mM of either MgCl₂, MnCl₂, CaCl₂, or 50 mM SDS) with Nsp1_FL and the different truncated protein versions, and the Y136F variant at a protein concentration of 5 μ M. In the assays presented in Figure S1, two protein concentrations were used, 1 μ M and 5 μ M. The reactions were started by the addition of 50 nM of the substrate and further incubated at 37°C. Aliquots of 4 μ L were withdrawn at the time points indicated in the respective figures, and the reactions were stopped by the addition of a formamide-containing dye supplemented with 10 mM of EDTA. A control reaction containing only the RNA or DNA substrate and the activity buffer (without the enzyme) was carried out in the same conditions. Reaction products were resolved in a 20% denaturant polyacrylamide gel (7M urea). Signals were visualized on a Phosphor Imaging system (TLA-5100 Series, Fuji). The quantification of the disappearance of both substrates along the time was determined using the ImageQuant (Cytiva), and represented using GraphPad Prism 8 software, as previously described (Salgueiro et al. 2024).

AUTHOR CONTRIBUTIONS

Bruno A. Salgueiro: Conceptualization; methodology; investigation; formal analysis; writing – original draft; writing – review and editing; data curation; resources. **Margarida Saramago:** Methodology; data curation; investigation; formal analysis; writing – original draft; writing – review and editing; resources. **Mark D. Tully:** Methodology; data curation; writing – review and editing; writing – original draft. **Cecília M. Arraiano:** Formal analysis; writing – original draft; writing – review and editing. **Elin Moe:** Methodology; conceptualization; data curation; investigation; resources; funding acquisition; writing – original draft; writing – review and editing. **Rute G. Matos:** Methodology; data curation; investigation; formal analysis; resources; writing – original draft; writing – review and editing. **Pedro M. Matias:** Conceptualization; supervision; funding acquisition; project administration; writing – review and editing; writing – original draft. **Célia V. Romão:** Conceptualization; methodology; investigation; formal analysis; supervision; funding acquisition; project administration; writing – original draft; resources; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its online Supporting Information.

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REFERENCES

- Abaeva IS, Arhab Y, Miścicka A, Hellen CUT, Pestova TV. In vitro reconstitution of SARS-CoV-2 Nsp1-induced mRNA cleavage reveals the key roles of the N-terminal domain of Nsp1 and the RRM domain of eIF3g. *Genes Dev.* 2023;37:844–60.
- Almeida MS, Johnson MA, Herrmann T, Geralt M, Wüthrich K. Novel β -barrel fold in the nuclear magnetic resonance structure of the replicase nonstructural protein 1 from the severe acute respiratory syndrome coronavirus. *J Virol.* 2007;81:3151–61.
- Banerjee AK, Blanco MR, Bruce EA, Honson DD, Chen LM, Chow A, et al. SARS-CoV-2 disrupts splicing, translation, and protein trafficking to suppress host defenses. *Cell.* 2020;183:1325–1339.e21.
- Borsatto A, Akkad O, Galdadas I, Ma S, Damfo S, Haider S, et al. Revealing druggable cryptic pockets in the Nsp1 of SARS-CoV-2 and other β -coronaviruses by simulations and crystallography. *eLife.* 2022;11:e81167.
- Burke JM, St Clair LA, Perera R, Parker R. SARS-CoV-2 infection triggers widespread host mRNA decay leading to an mRNA export block. *RNA.* 2021;27:1318–29.
- Clark LK, Green TJ, Petit CM. Structure of nonstructural protein 1 from SARS-CoV-2. *J Virol.* 2021;95:e02019–20.
- de Wit E, van Doremalen N, Falzarano D, Munster VJ. SARS and MERS: recent insights into emerging coronaviruses. *Nat Rev Microbiol.* 2016;14:523–34.
- Deweese JE, Osheroff N. The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing. *Nucleic Acids Res.* 2009;37:738–48.
- Ghaleh SS, Rahimian K, Mahmanzar M, Mahdavi B, Tokhanbigli S, Sisakht MM, et al. SARS-CoV-2 non-structural protein 1(NSP1) mutation virulence and natural selection: evolutionary trends in the six continents. *Virus Res.* 2023;323:199016.
- Gomez GN, Abrar F, Dodhia MP, Gonzalez FG, Nag A. SARS coronavirus protein nsp1 disrupts localization of Nup93 from the nuclear pore complex. *Biochem Cell Biol.* 2019;97:758–66.
- Gordon DE, Jang GM, Bouhaddou M, Xu J, Obernier K, White KM, et al. A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature.* 2020;583:459–68.
- Gustin KM, Guan B-J, Dziduszko A, Brian DA. Bovine coronavirus nonstructural protein 1 (p28) is an RNA binding protein that binds terminal genomic *cis*-replication elements. *J Virol.* 2009;83:6087–97.
- Hasoksuz M, Kiliç S, Saraç F. Coronaviruses and SARS-COV-2. *Turk J Med Sci.* 2020;50:549–56.
- Kaub J, Akinselure T, von Soosten L, Santoni G, Thorn A. Structural biology of SARS-CoV-2 leader protein (nsp1). *Crystallogr Rev.* 2024;1–17.
- Kieffer J, Brennich M, Florial J-B, Oscarsson M, De Maria AA, Tully M, et al. New data analysis for BioSAXS at the ESRF. *J Synchrotron Radiat.* 2022;29:1318–28.
- Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. *New Engl J Med.* 2003;348:1953–66.
- Kumar A, Kumar A, Kumar P, Garg N, Giri R. SARS-CoV-2 NSP1 C-terminal (residues 131–180) is an intrinsically disordered region in isolation. *Curr Res Virol Sci.* 2021;2:100007.
- Lai C-C, Shih T-P, Ko W-C, Tang H-J, Hsueh P-R. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. *Int J Antimicrob Agents.* 2020;55:105924.
- Libich DS, Baudin A. Order and disorder bound together in SARS-CoV-2 Nsp1 suppress host translation. *Structure.* 2023;31:121–2.
- Lokugamage KG, Narayanan K, Huang C, Makino S. Severe acute respiratory syndrome coronavirus protein nsp1 is a novel eukaryotic translation inhibitor that represses multiple steps of translation initiation. *J Virol.* 2012;86:13598–608.
- Ma S, Damfo S, Lou J, Pinotsis N, Bowler MW, Haider S, et al. Two ligand-binding sites on SARS-CoV-2 non-structural protein 1 revealed by fragment-based X-ray screening. *Int J Mol Sci.* 2022;23:12448.
- Meisburger SP, Taylor AB, Khan CA, Zhang S, Fitzpatrick PF, Ando N. Domain movements upon activation of phenylalanine hydroxylase characterized by crystallography and chromatography-coupled small-angle X-ray scattering. *J Am Chem Soc.* 2016;138:6506–16.
- Mendez AS, Ly M, González-Sánchez AM, Hartenian E, Ingolia NT, Cate JH, et al. The N-terminal domain of SARS-CoV-2 nsp1 plays key roles in suppression of cellular gene expression and preservation of viral gene expression. *Cell Rep.* 2021;37:109841.
- Micsonai A, Moussong É, Murvai N, Tantos Á, Tőke O, Réfrégiers M, et al. Disordered-ordered protein binary classification by circular dichroism spectroscopy. *Front Mol Biosci.* 2022a;9:863141.
- Micsonai A, Moussong É, Wien F, Boros E, Vadász H, Murvai N, et al. BeStSel: webserver for secondary structure and fold prediction for protein CD spectroscopy. *Nucleic Acids Res.* 2022b;50:W90–8.
- Min Y-Q, Mo Q, Wang J, Deng F, Wang H, Ning Y-J. SARS-CoV-2 nsp1: bioinformatics, potential structural and functional features, and implications for drug/vaccine designs. *Front Microbiol.* 2020;11.
- Mundle ST, Fattal MH, Melo LF, Coriolan JD, O'Regan NE, Strauss PR. Novel role of tyrosine in catalysis by human AP endonuclease 1. *DNA Repair (Amst).* 2004;3:1447–55.

- Nakagawa K, Makino S. Mechanisms of coronavirus Nsp1-mediated control of host and viral gene expression. *Cells*. 2021;10:300.
- Parker W, Song PS. Protein structures in SDS micelle-protein complexes. *Biophys J*. 1992;61:1435–9.
- Sajidah ES, Lim K, Wong RW. How SARS-CoV-2 and other viruses build an invasion route to hijack the host nucleocytoplasmic trafficking system. *Cells*. 2021;10:1424.
- Salgueiro BA, Saramago M, Tully MD, Issoglio F, Silva STN, Paiva ACF, et al. SARS-CoV2 Nsp1 is a metal-dependent DNA and RNA endonuclease. *Biometals*. 2024;37:1127–46.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9:676–82.
- Schubert K, Karousis ED, Jomaa A, Scaiola A, Echeverria B, Gurzeler L-A, et al. SARS-CoV-2 Nsp1 binds the ribosomal mRNA channel to inhibit translation. *Nat Struct Mol Biol*. 2020;27:959–66.
- Semper C, Watanabe N, Savchenko A. Structural characterization of nonstructural protein 1 from SARS-CoV-2. *iScience*. 2021;24:101903.
- Shehata SI, Parker R. SARS-CoV-2 Nsp1 mediated mRNA degradation requires mRNA interaction with the ribosome. *RNA Biol*. 2023;20:444–56.
- Shi M, Wang L, Fontana P, Vora S, Zhang Y, Fu T-M, et al. SARS-CoV-2 Nsp1 suppresses host but not viral translation through a bipartite mechanism. *SSRN Electron J*. 2020.
- Simeoni M, Cavinato T, Rodriguez D, Gatfield D. I(nsp1)ecting SARS-CoV-2–ribosome interactions. *Commun Biol*. 2021;4:715.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985;150:76–85.
- Sosnowski P, Tidu A, Eriani G, Westhof E, Martin F. Correlated sequence signatures are present within the genomic 5'UTR RNA and NSP1 protein in coronaviruses. *RNA*. 2022;28:729–41.
- Tanaka T, Kamitani W, DeDiego ML, Enjuanes L, Matsuura Y. Severe acute respiratory syndrome coronavirus nsp1 facilitates efficient propagation in cells through a specific translational shutoff of host mRNA. *J Virol*. 2012;86:11128–37.
- Tardivat Y, Sosnowski P, Tidu A, Westhof E, Eriani G, Martin F. SARS-CoV-2 NSP1 induces mRNA cleavages on the ribosome. *Nucleic Acids Res*. 2023;51:8677–90.
- Terada Y, Kawachi K, Matsuura Y, Kamitani W. MERS coronavirus nsp1 participates in an efficient propagation through a specific interaction with viral RNA. *Virology*. 2017;511:95–105.
- Thoms M, Buschauer R, Ameismeier M, Koepke L, Denk T, Hirschenberger M, et al. Structural basis for translational shut-down and immune evasion by the Nsp1 protein of SARS-CoV-2. *Science*. 2020;369:1249–55.
- Tidu A, Janvier A, Schaeffer L, Sosnowski P, Kuhn L, Hamann P, et al. The viral protein NSP1 acts as a ribosome gatekeeper for shutting down host translation and fostering SARS-CoV-2 translation. *RNA*. 2021;27:253–64.
- Tomter AB, Zoppellaro G, Schmitzberger F, Andersen NH, Barra A-L, Engman H, et al. HF-EPR, Raman, UV/VIS light spectroscopic, and DFT studies of the ribonucleotide reductase R2 tyrosyl radical from Epstein-Barr virus. *PLoS One*. 2011;6:e25022.
- Tully MD, Kieffer J, Brennich ME, Cohen Aberdam R, Florial JB, Hutin S, et al. BioSAXS at European Synchrotron Radiation Facility—extremely brilliant source: BM29 with an upgraded source, detector, robot, sample environment, data collection and analysis software. *J Synchrotron Radiat*. 2023;30:258–66.
- Tully MD, Tarbouriech N, Rambo RP, Hutin S. Analysis of SEC-SAXS data via EFA deconvolution and Scatter. *J Vis Exp*. 2021.
- Tulumello DV, Deber CM. SDS micelles as a membrane-mimetic environment for transmembrane segments. *Biochemistry*. 2009;48:12096–103.
- Vora SM, Fontana P, Mao T, Leger V, Zhang Y, Fu T-M, et al. Targeting stem-loop 1 of the SARS-CoV-2 5' UTR to suppress viral translation and Nsp1 evasion. *Proc Natl Acad Sci*. 2022;119:e2117198119.
- Wang Y, Kirkpatrick J, Lage S, Carlomagno T. Structural insights into the activity regulation of full-length non-structural protein 1 from SARS-CoV-2. *Structure*. 2023;31:128–137.e5.
- Wathelet MG, Orr M, Frieman MB, Baric RS. Severe acute respiratory syndrome coronavirus evades antiviral signaling: role of nsp1 and rational design of an attenuated strain. *J Virol*. 2007;81:11620–33.
- Wolff G, Limpens RWAL, Zevenhoven-Dobbe JC, Laugks U, Zheng S, de Jong AWM, et al. A molecular pore spans the double membrane of the coronavirus replication organelle. *Science*. 2020;369:1395–8.
- Yang W. Nucleases: diversity of structure, function and mechanism. *Q Rev Biophys*. 2011;44:1–93.
- Yuan S, Peng L, Park JJ, Hu Y, Devarkar SC, Dong MB, et al. Non-structural protein 1 of SARS-CoV-2 is a potent pathogenicity factor redirecting host protein synthesis machinery toward viral RNA. *Mol Cell*. 2020;80:1055–1066.e6.
- Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *New Engl J Med*. 2012;367:1814–20.
- Zhang K, Miorin L, Makio T, Dehghan I, Gao S, Xie Y, et al. Nsp1 protein of SARS-CoV-2 disrupts the mRNA export machinery to inhibit host gene expression. *Sci Adv*. 2021;7:eabe7386.
- Zhao K, Ke Z, Hu H, Liu Y, Li A, Hua R, et al. Structural basis and function of the N terminus of SARS-CoV-2 nonstructural protein. *Microbiol Spectr*. 2021;1:9.
- Zimmermann L, Zhao X, Makroczynova J, Wachsmuth-Melm M, Prasad V, Hensel Z, et al. SARS-CoV-2 nsp3 and nsp4 are minimal constituents of a pore spanning replication organelle. *Nat Commun*. 2023;14:7894.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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