Atomic-Resolution Structure of SARS-CoV-2 Nucleocapsid Protein N-Terminal Domain

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ABSTRACT: The nucleocapsid (N) protein is one of the four structural proteins of the SARS-CoV-2 virus and plays a crucial role in viral genome organization and, hence, replication and pathogenicity. The N-terminal domain (NNTD) binds to the genomic RNA and thus comprises a potential target for inhibitor and vaccine development. We determined the atomic-resolution structure of crystalline NNTD by integrating solid-state magic angle spinning (MAS) NMR and X-ray diffraction. Our combined approach provides atomic details of protein packing interfaces as well as information about flexible regions as the N- and C-termini and the functionally important RNA binding, β-hairpin loop. In addition, ultrafast (100 kHz) MAS 1H-detected experiments permitted the assignment of side-chain proton chemical shifts not available by other means. The present structure offers guidance for designing therapeutic interventions against the SARS-CoV-2 infection.

INTRODUCTION

SARS-CoV-2, a positive-sense single-stranded RNA virus from the β-coronavirus family,1 is the causative agent of the COVID-19 pandemic that killed millions of people and brought the world economy to a grinding halt.2,3 The SARS-CoV-2 genome encodes four structural proteins: spike (S) glycoprotein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein.4,5 All play crucial roles in the viral life cycle and pathogenicity, including host immunity evasion.6 Due to its important role in genome packaging and ribonucleoprotein (RNP) formation, the N protein represents a potential target for therapeutic interventions.7−9

The N protein comprises two folded domains, the N-terminal (NNTD, residues 40−174) and C-terminal (NTD) domains, connected by an ~70 amino acid linker region that contains a 13-residue serine/arginine motif, as well as extensive intrinsically disordered regions (IDRs) at the N- and C-termini10−15 (Figure 1a). All domains including the NNTD play an important role in the RNA genome interaction.16−18

Several structures of β-coronavirus NNTD domains have been reported, all of which possess the same architecture, resembling a right hand.19−24 The core structure is made of a four-stranded antiparallel β-sheet, the palm, from which the β2, β3 hairpin prominently protrudes. It contains several basic residues, and this basic finger and the palm have been implicated in RNA binding.25 The loop connecting β2 and β3 is flexible, in agreement with the missing density in this region of most X-ray structures (see below).19,26 The N-terminal disordered tail projects outward and may contribute to RNA binding.19

Here, we report the atomic-resolution structure of crystalline NNTD, determined by combining X-ray crystallography and solid-state magic angle spinning (MAS) NMR spectroscopy. The protein crystallized in the P212121 space group with four chains in the asymmetric unit, and the X-ray structure was solved at a 1.7 Å resolution. The MAS NMR structure of an individual NNTD chain, at 0.7 Å rmsd resolution, was determined using a single crystalline U−13C,15N−NNTD sample, based on 2968 nonredundant 13C−13C, 15N−13C, and 15N−1H distance restraints. Several inter-chain contacts were identified in 13C−13C correlation experiments, both for chains in the asymmetric unit as well as across asymmetric units. Side-chain proton chemical shifts were assigned from high-frequency (100 kHz) MAS NMR correlation experiments and provided important structural information, such as the tautomeric state of the H107 residue. Our results illustrate the
power of integrating orthogonal structural techniques, here MAS NMR and X-ray diffraction, for assessing details of protein conformations. The atomic-resolution structure of crystalline NNTD reported here will guide the development of small-molecule inhibitors and biologics for treatment as well as biosensors for the detection of SARS-CoV-2 infection.27

Figure 1. Domain delineation, amino acid sequence, and magic angle spinning (MAS) NMR spectra used for resonance assignment of SARS-CoV-2 NNTD. (a) Top: domain organization of SARS-CoV-2 nucleocapsid (N) protein; N-terminal domain (NNTD) and C-terminal domain (NCTD). Bottom: NNTD primary sequence and β-strands (blue arrows); residues 2–136 (black) in the current NNTD construct (this work) correspond to residues 40–174 (gray) in the full-length N protein. (b) Selected regions of 1H-detected two-dimensional (2D) (H)NH HETCOR and (H)CH HETCOR spectra of U–13C,15N–NNTD. The expansions around the A52 and T38 cross-peaks in (H)NH and (H)CH spectra depict one-dimensional (1D) slices to illustrate the line widths in the two frequency dimensions. (c) Aliphatic region of the 2D CORD spectrum (25 ms mixing time). (d) Sequential assignments for the D44–G47 stretch of residues are illustrated with representative strips of 2D NCACX (gray), NCOCX (blue), 1H-detected three-dimensional (3D) (H)CANH (black), and (H)CONH (pale blue) spectra. (e) Selected strips from the 1H-detected 3D (H)CCH spectrum for residues T77 and P42 illustrating 13C and 1H side-chain resonance assignments. CORD, NCACX, and NCOCX spectra were recorded at an MAS frequency of 14 kHz; (H)CANH and (H)CONH spectra were acquired at an MAS frequency of 60 kHz; and HETCOR and (H)CCH spectra were acquired at an MAS frequency of 100 kHz. The number of scans and the number points in the direct and indirect dimensions are as follows: 2D (H)NH HETCOR - 32 scans, 1024 t2 points, 1034 t1 points; 2D (H)CH HETCOR - 64 scans, 1024 t2 points, 2310 t1 points; 2D CORD - 192 scans, 2048 t2 points, 840 t1 points; 2D NCACX - 2048 scans, 2048 t2 points, 96 t1 points; 2D NCOCX - 1536 scans, 3072 t2 points, 96 t1 points; 3D (H)CANH - 48 scans, 2048 t2 points, 112 13C t1 points, 32 15N t1 points; 3D (H)CONH - 32 scans, 2048 t2 points, 112 13C t1 points, 32 15N t1 points; and 3D (H)CCH - 8 scans, 1024 t2 points, 264 13C t1 points, 264 13C t1 points.
RESULTS

Resonance Assignments. Chemical shift assignments and distance restraints for N$^{\text{NTD}}$ structure calculation were obtained using a single sample of fully protonated crystalline U$^{\text{−}13\text{C},15\text{N}−}\text{NNTD}$ comprising residues 40−174 (current construct residues 2−136; Figure 1a; see the Materials and Methods section for experimental details). A total of eleven 2D and three 3D$^{1\text{H}}$- and $^{13\text{C}}$-detected high-frequency (100 and 60 kHz) MAS NMR experiments were recorded (Figure 1b−e and Supporting Information Table S1). The spectra are of remarkably high resolution, with line widths as narrow as 35 Hz for $^{15\text{N}}$, 48 Hz for $^{13\text{C}}$, and 174 Hz for $^{1\text{H}}$ (Figure 1b).

2D CORD,$^{28}$ NCACX, and NCOCX at a 25 ms mixing time, as well as $^{1\text{H}}$-detected 2D (H)NH HETCOR, 3D (H)CANH, and (H)CONH spectra (Figure 1c,d), were used for sequential backbone assignments, and $^{13\text{C}}$ and $^{15\text{N}}$ chemical shifts are complete for 128 of 136 residues. For five residues, F28, P84, P113, P124, and E136, partial backbone chemical shift assignments were obtained, and, for 119 residues, backbone amide proton (HN) chemical shifts were assigned. The resonances of the first two residues, R2 and R3, are missing in the spectra, likely due to disorder. Overall, good agreement is observed between $^{1\text{H}}$ and $^{13\text{C}}$ backbone chemical shifts determined in this work and those reported previously from solution NMR.$^{25,29}$ MAS NMR assignments for a representative stretch of residues D44−G47 are illustrated in Figure 1d.

Side-chain $^{13\text{C}}$ chemical shifts and inter-residue correlations were obtained from 2D NCACX, NCOCX, and CORD spectra, the latter acquired with 25, 100, and 500 ms mixing times (Figures 1c and 2a). High spectral resolution permitted the unambiguous assignment of numerous cross-peaks, including those corresponding to aliphatic-to-aromatic (left panel) and aliphatic-to-aliphatic (right panel) side-chain correlations (Figure 2a). To determine side-chain and backbone $^{1\text{H}}$ chemical shifts, a 3D (H)CCH correlation experiment was recorded at an MAS frequency of 100 kHz (Figure 1e). In conjunction with spectra acquired at an MAS frequency of 60 kHz, 84 side-chain proton resonances for 71 residues and $^{1\text{H}}$α resonances for 65 residues were assigned. For 11 Ala, 3 Val, 4 Ser, and 1 His residues, complete $^{13\text{C}},^{15\text{N}},$ and $^{1\text{H}}$ backbone and side-chain chemical shifts were obtained. Overall, assignments for 132 residues were attained (Figure S1) on the basis of 3728 cross-peaks in various spectra (Table 1). All chemical shifts are summarized in Table S2 of the Supporting Information.

Gratifyingly, many side-chain protons of aromatic residues could be unambiguously assigned from the $^{1\text{H}}$-detected 100 kHz MAS NMR spectra (Figure 1b). For example, for W70 and W94, located in the $\beta$-sheet core and assumed to be involved in RNA binding, side-chain protons were assigned fully (W70) or partially (W94). Moreover, the tautomeric state of H107 was determined (see below).

Structure of a Single N$^{\text{NTD}}$ Chain Determined by MAS NMR. The structure of an N$^{\text{NTD}}$ single chain was calculated using 2968 nonredundant distance and 101 $\phi/\psi$ torsion angle restraints. Of these, 2197 are unambiguous $^{13\text{C}}−^{13\text{C}}$, 763 are
Like all coronavirus N\textsuperscript{NTD} structures,\textsuperscript{16,19,24–26,31,32} the MAS NMR-derived structure exhibits the overall shape of a right hand, made up of a four-stranded \(\beta\)-sheet, comprising \(\beta\)1 (L18–T19), \(\beta2\) (I46–R55), \(\beta3\) (D65–Y74), and \(\beta4\) (I92–A96). At its center, a long \(\beta\)-hairpin protrudes out from the palm (Figure 2c). The irregular regions at the N- and C-termini exhibit well-defined backbone and side-chain orientations in the MAS NMR structure, except for the first eight amino acids (R2–N9) and the last residue (E136) (see Figures S2 and S3 of the Supporting Information). The lack of long-range inter-residue distance restraints for the N-terminal tail residues (P4–N9) and \(\beta\)-hairpin loop (I56–K64) suggests that they are dynamic (Figure 2b). The precision of the single-chain MAS NMR structure is 0.7 ± 0.2 \(\text{Å}\), as measured by the pairwise atomic backbone rmsd for the 10 lowest-energy structures (excluding the disordered N-terminal tail, residues R2–N9) (Table 2 and Figure S3 of the Supporting Information).

**X-ray Crystal Structure of the N\textsuperscript{NTD}**. The protein crystallized in the \(P2_12_12_1\) orthorhombic space group with four monomers (chains A–D) in the asymmetric unit (Figures 3a and S4a of the Supporting Information). Two views of the four chains are provided in Figure 3a, and chain A is depicted in Figure 3b. Details of the \(\beta2\), \(\beta3\) hairpin and loop region, as well as the difference electron density map, are shown in Figure 3c. Complete statistics for X-ray data collection, phasing, and refinement are provided in Table 3. The average pairwise rmsd value between the four chains in the asymmetric unit is 0.5 ± 0.1 \(\text{Å}\) for the backbone atoms (excluding common missing residues in all four chains, R2–N9, Q20–D25, R57–P68, and P124–E136) (Figure S5b and Table S3 of the Supporting Information). A positively charged region, comprising arginine residues in the \(\beta\)-sheet (R50, R51, R54, R55, R69) and at the tip of the \(\beta\)-hairpin finger (R57, K62), possibly might contribute to RNA binding (Figure S4c of the Supporting Information).\textsuperscript{19,25}

Interesting details about the intratetramer interfaces in the crystal can be noted in the structure, with five unique types of contacts formed by the residues within the tetramer (Figure 4a). Specifically, (i) the A–B interface comprises several residues (T18, H21, I36, R54, R56–G59, K64, L66, S67, V120, Q122, Y134, and A135) from both chain A and chain B; (ii) the A–C interface is very small and involves residues G59 and D60 of chain C contacting K131 of chain C; (iii) the B–C interface comprises several residues, such as R30–Q32, P42, D43, E98–L101, and P124–T127 of the chain B palm region, which are in contact with the C-terminal tail (residues L121–L129, K131, G132, Y134, A135), as well as H21, G22, and K23 of chain C; (iv) the B–D interface packs the palm regions of chains B and D against each other; and (v) the D–C interface comprises residues I56 and P113–A117 of chain D and T16, H21, A117, A118, V120, and Q122 of chain C.

The X-ray and the MAS NMR structures of the individual chains are in good agreement, with a backbone rmsd of 1.1 \(\text{Å}\) between the X-ray structure (averaged over the four chains in the asymmetric unit) and the MAS NMR structure (averaged over the ensemble of 10 lowest-energy structures) (Figure S5 and Table S3 of the Supporting Information). Upon exclusion of chain D, which possesses the highest degree of disorder in the X-ray structure, the corresponding value becomes 0.7 \(\text{Å}\). The average pairwise backbone rmsd between the four chains in the X-ray structure and within the ensemble of 10 lowest-energy MAS NMR structures are, both, 0.5 ± 0.1 \(\text{Å}\) (Table S3).

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**Table 1. Summary of Samples and the Number of Assigned Peaks**

| Sample | no. of assigned peaks* |
|--------|------------------------|
| U–\(^{13}\text{C}\), \(^{15}\text{N}\)–\(^{13}\text{C}\) (MAS NMR) | 3728 |
| intraresidue | 1943 |
| sequential \((l - j = 1)\) | 495 |
| medium range \((1 < l - j < 5)\) | 306 |
| long range \((l - j ≥ 5)\) | 972 |
| total assigned peaks (MAS NMR) | 3728 |

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**Table 2. Summary of MAS NMR Restraints and Structure Statistics**

| MAS NMR distance restraints | \(^{13}\text{C}–^{13}\text{C}\) | \(^{15}\text{N}–^{13}\text{C}\) | \(^{13}\text{C}–^{15}\text{N}\) |
|-----------------------------|-----------------|-----------------|-----------------|
| unambiguous | 2197 | 763 | 4 |
| intraresidue | 807 | 505 | 0 |
| sequential \((l - j = 1)\) | 119 | 258 | 4 |
| medium range \((1 < l - j < 5)\) | 303 | 0 | 0 |
| long range \((l - j ≥ 5)\) | 968 | 0 | 0 |
| ambiguous | 4 |
| total number of restraints assigned | 2968 (21.8 restraints per residue) |

| MAS NMR Dihedral Angle Restraints |
|----------------------------------|
| \(\Phi\) | 101 |
| \(\Psi\) | 101 |

| Structure Statistics from 10 Lowest-Energy Subunits |
|---------------------------------|
| violations \((\text{mean} ± \text{sd})\) |
| distance restraints ≥ 7.2 \(\text{Å}\) \((\text{Å})\) | 0.144 ± 0.001 |
| dihedral angle restraints ≥ 5° \((\text{deg})\) | 1.528 ± 0.137 |
| max. distance restraint violation \((\text{Å})\) | 1.254 |
| max. dihedral angle restraint violation \((\text{deg})\) | 17.267 |
| deviations from idealized geometry |
| bond lengths \((\text{Å})\) | 0.008 ± 0.000 |
| bond angles \((\text{deg})\) | 0.774 ± 0.012 |
| improper pairs \((\text{deg})\) | 0.516 ± 0.016 |
| average pairwise rmsd \((\text{Å})\)* |
| backbone \((\text{N}, \text{C}_\alpha, \text{C}'\)) | 0.7 ± 0.2 |
| heavy | 1.2 ± 0.1 |

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*Cross-peaks present in different experiments are counted only once.

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Supporting Information). The number of restraints per residue is plotted in Figure 2b. The 10 lowest-energy MAS NMR structures in the structural ensemble and an average structure of a single chain of N\textsuperscript{NTD} are shown in Figures 2c and S3 of the Supporting Information, respectively. All MAS NMR distance restraints are summarized in Table 2. With nearly 22 restraints/residue on average, the N\textsuperscript{NTD} structure determined in this study represents a notable technical advance being one of only two MAS NMR structures of proteins larger than 100 residues per chain determined using more than 20 restraints/residue and reaching the maximum accuracy and precision attained for MAS NMR protein structures\textsuperscript{30} (see below).
of the Supporting Information). Side-chain conformations for most residues in all four chains of the X-ray structure vary little, except for residues N10, R30, R55, and I56, which exhibit major differences (Figure S6 of the Supporting Information). Unlike the MAS NMR structure, which was determined using distance restraints or/and chemical shifts for most residues, except R2, R3, and E136, density is either missing or very weak for residues R2–N9 and E136 in all chains and for residues Q20–D25, R57–P68 and P124–E136 in chain D.

In addition to the five intratetramer interfaces, each tetramer (arbitrarily designated as “tetramer 0”) contacts 10 neighboring tetramers (tetramers 1–10) resulting in four distinct intertetramer interfaces, classified by symmetry operators. The nomenclature for a specific chain (A) in a tetramer (0) is denoted as 0A. The packing of tetramers in the crystallographic lattice is depicted in Figure 4b. Intertetramer interface 1 is formed by two tetramers, 1 and 2, adjacent to tetramer 0. This interface comprises residues G82–P84 and A87–K89 of chains C (0C) and D (0D) of tetramer 0 and equivalent residues G82–P84 and A87–K89 in 2C and 1C, respectively (colored light gray in Figure 4b(i)). Intertetramer interface 2 is formed by four tetramers, 3, 4, 5, and 6 (colored green in Figure 4b(ii)) around tetramer 0. Several residues in 0A (N10–W14, T16, T38–S41, D44, R50–R57, K62, M63, L66, R69, Y71, Y73, P79, D106–V120), 0B (N10–S13, T16, T38–S40, R50–R57, G58–D60, M63, L66, R69, Y71, Y73, P79, G82–K89, R111–V120, Q122), 0C (N10, T11, W14, R30, N37–I46, Y48, L75–A87, V95–I119), and 0D (N10, T11, W14, N39, S41–D44, I46, Y48, L75–A87, A96–N116) are involved in crystallographically inequivalent interfaces (see Table S4).

Intertetramer interface 3 is formed by two tetramers, 7 and 8 (colored pink in Figure 4b(iii)), adjacent to tetramer 0. Residues H21–D25, K27, P29–Q32, A96–G99, and Q122–A135 in 0A, Q20–K23, R57, K62, H66–P68, and Y134 in 0B, V120, Q122, R57, K62, M63, and D65–L66 in 0C, and I36 and V120–L123 of 0D have contacts with residues comprising tetramers 7 and 8. Intertetramer interface 4 comprises two tetramers, 9 and 10 (colored blue in Figure 4b(iv)), adjacent to tetramer 0. The residues involved in this interface are E80, G82–P84, and A87–K89 of 0A and 9A and W14, D106–R112 in 10B and 0B, respectively. All intra- and intertetramer contacts for each NNTD residue are summarized in Table S4.

These unique intra- and intertetramer interfaces are reflected in distinct correlations in the MAS NMR spectra. In the (H)NH spectrum recorded at an MAS frequency of 100 kHz, multiple resolved peaks or broad (unresolved) peaks are observed for residues that are found in several different local environments, with 15N peak widths of ~85–110 Hz (Figure 5b), whereas those for amino acids in single conformations are ~40–60 Hz (Figure 5a,c). Examples of correlations corresponding to intratetramers A–B, A–C, and B–D interfaces, as well as to the intertetramer interfaces, are shown in Figure 4a,b, bottom panels.

One notable example of unique intertetramer contacts, evidenced by multiple cross-peaks in the MAS NMR spectra, is seen for the H21–Y134 pair of residues (Figure 4a, bottom panel). It is evident from the X-ray structure that the interchain distances are much shorter than the intrachain ones (3.5–4.6 and 7.0–8.0 Å, respectively); hence, only interchain correlations are expected in the spectra (Figure 6). Another interesting example involves residues D60 and M63 in the β2,
Table 3. X-ray Data Collection and Refinement Statistics
da

| SARS-CoV-2 NNTD |  |
|-----------------|---|
| **Data Collection** |  |
| wavelength (Å) | 0.9794 |
| space group | P2₁,2₁,2₁ |
| cell dimensions | a, b, c (Å) 58.76, 92.76, 95.59 |
| α, β, γ (deg) | 90, 90, 90 |
| resolution range (Å) | 37–170 |
| Rmerge or Rmerge | 0.024(0.65) |
| I/σ(I)-CC1/2 | 27.5(1.1)-99(42) |
| completeness (%) | 98.9(98.2) |
| **Refinement** |  |
| refinement program | COOT |
| resolution range (Å) | 37–170 |
| no. of reflections | 59,316 |
| Rmerge/Refl | 24.4/28.5 |
| no. of nonhydrogen atoms |  |
| protein | 3642 |
| solvent (water) | 650 |
| B-factors |  |
| protein | 48.5 |
| solvent (water) | 42 |
| Rms deviations |  |
| bond lengths (Å) | 0.003 |
| bond angles (deg) | 0.68 |
| PDB ID | 7UW3 |

“Molecular replacement. bValues in parentheses are for the highest-resolution shell.

β3-hairpin loop, for which intra- and intertetramer correlations were identified based on the following considerations: (i) D60 from chain A with no intertetramer contacts has a unique intratetramer correlation with K131 at the A–C interface. In contrast, there are no intratetramer correlations involving D60 from other chains with K131, and only intertetramer correlations are present; (ii) M63 has no intratetramer interactions; (iii) M63 from chains A and B is in close proximity to N39 in the symmetry-related tetramer interface 2; and (iv) M63 from chain C forms contacts with A135 across the tetramer interface 3 (Figure 4b, bottom panel, and Figure 7).

In addition to the intra- and intertetramer contacts discussed above, we also observed multiple conformers for many residues, as is evident from their distinct backbone and side-chain chemical shifts. For example, T77 exhibits several resolved resonances with unique chemical shifts each (Figure 4c, left panel). A unique T77C′′/A117C′ cross-peak is found for one of the two conformers (designated as conformer b, T77b) in the 100 ms mixing time CORD spectrum. This correlation is missing for the second conformer, T77b. Both conformers exhibit significant chemical shift differences (ΔN = 1.4 ppm, ΔC′ = 0.3 ppm, ΔC′′ = 0.3 ppm, ΔC = 0.6 ppm), consistent with distinct local environments for T77. Indeed, in the X-ray structure, T77 in 0A and 0B has no intra- or intertetramer contacts within 7 Å, consistent with this conformer being T77b. In contrast, T77 in chains 0C and 0D exhibits intertetramer contacts with A117 from chains 4A and 3A respectively, therefore suggesting that these resonances correspond to the T77a conformer (Figure 4c, right panels). Likewise, at least two distinct conformations are seen for A52, whose backbone chemical shifts are different (ΔN = 2.0 ppm, ΔC = 0.4 ppm), suggesting that they exist in unique local environments (Figure S7, top panel, Supporting Information). This finding is fully consistent with the X-ray structure (Figure S7, bottom panel, Supporting Information), where A52 in 0A and 0B forms intertetramer contacts with D43 and L101 from chains 6C and 5D, respectively, while A52 in chains 0C and 0D forms contacts with E24 and K27 from chains 8A and 0b, respectively.

Tautomeric State of Histidine-107 in the Crystal. In the 1H-detected 2D (H)NH spectrum acquired with a CP contact time of 0.3 ms, H107 gives rise to two distinct 15N=H H2 cross-peaks at δ(15N) = 170.2 ppm/δ(H) = 12.4 ppm and δ(15N) = 170.4 ppm/δ(H) = 12.6 ppm (Figures 1b and 8a). In 2D (H)CH spectra, multiple C′1−H1 and C′2−H2 correlations are also observed (Figure 8a). Taken together, these results suggest the presence of local heterogeneity around H107, consistent with two distinct local environments seen in the X-ray crystal structure (Table S4 of the Supporting Information).

The tautomeric state of H107 was ascertained by a 1H-detected 2D (H)NH experiment acquired with a CP contact time of 4 ms (Figure 8a). The cross-peaks at δ(1H) = 7.8 ppm, δ(15N) = 170 ppm are assigned to the N-H tautomer (Figures 8b and 8a). The N-H tautomer is present in two states, with H107 in close proximity to N39 and A135 across the tetramer interface 3 (Figure S8 of the Supporting Information), where A52 in 0A and 0B forms intertetramer contacts with D43 and L101 from chains 6C and 5D, respectively, while A52 in chains 0C and 0D forms contacts with E24 and K27 from chains 8A and 0b, respectively.

The chemical shift of the H2 resonance at δ(1H) = 12.5 ppm suggests that the H107 imidazole may be involved in hydrogen bonding or close to a negatively charged group. The presence of a 15N=H2−H2 correlation in the (H)NH spectrum acquired with a 0.3 ms CP contact time also suggests a short nitrogen−hydrogen distance, possibly a directly bonded N-H tautomer. Further evidence comes from a solution HMBC spectrum recorded at the pH of the crystallization (pH 6.3) (Figure S8 of the Supporting Information), where N2=H4 and N2−H2 correlations were observed at δ(15N) = 170 ppm/δ(H) = 7.7 ppm and δ(15N) = 170 ppm/δ(H) = 6.9 ppm, respectively. The N4=H4 correlation at δ(15N) = 243.4 ppm/δ(H) = 7.7 ppm is consistent with the H107 being the N2=H tautomer.

The deshielded H2 resonance at δ(1H) = 12.5 ppm suggests that the H107 imidazole may be involved in hydrogen bonding or close to a negatively charged group. The presence of a 15N=H2−H2 correlation in the (H)NH spectrum acquired with a 0.3 ms CP contact time also suggests a short nitrogen−hydrogen distance, possibly a directly bonded N2−H. Indeed, in the X-ray crystal structure, H107 is in close proximity to D44 and the interatomic H107N2−D44O5.6,2 distances are 2.9, 2.9, 3.2, and 3.4 Å in chains A, B, C, and D, respectively (Figure 8b). Taken together, these data indicate that the H107N2−H is close to the carboxylate side chain of D44.

DISCUSSION

The NTD domain of the SARS-CoV-2 virus N protein has been previously structurally characterized by X-ray crystallography and solution NMR. Here, we present a structure that was determined by integrating MAS NMR and X-ray diffraction, providing important novel findings of distinct conformers, made possible by the remarkably high resolution of the MAS NMR spectra. The structural heterogeneity of the NNTD is an outcome of crystallization, as seen in other NNTD crystal structures, and underscores the ability of the protein to form multiple types of contacts involving distinct conformers with unique local environments.

From the technical standpoint, the current study represents a notable advance for determining protein structures by MAS
NMR since a single sample of only 3.6 mg of U−$^{13}$C,15N-labeled NNTD packed in a 1.3 mm MAS rotor was sufficient to obtain all necessary spectra. The same sample (∼0.5 mg) was subsequently packed in a 0.7 mm MAS rotor for 1H-detected experiments at 100 kHz MAS and 20.0 T, and these additional experiments yielded unique information on the side-chain protons. Resonances for 98% of all amino acids were assigned, and a large number of correlations corresponding to 2968 distance restraints, including 968 long-range restraints, were obtained from 14 2D and 3D data sets. As a result, no preparations of isotopically diluted samples were necessary. At 21.8 restraints per residue, the NNTD single-chain structure reported here is one of the highest precision and accuracy MAS NMR structures determined to date.

**Figure 4.** Interchain interfaces and crystal packing in the NNTD structure. (a) Intratetramer interfaces. Top and middle: five unique interchain interfaces in the asymmetric unit of NNTD crystal; chain A (gray), chain B (purple), chain C (cyan), and chain D (orange). Interface residues are in yellow stick representation. (b) Intertetramer interfaces. Top-left: each single tetramer (numbered 0) forms intertetramer interfaces with 10 neighboring tetramers (1−10). Intertetramer interface residues are colored yellow. Top-right and middle-right: four unique intertetramer interfaces are formed based on symmetry operation. The nomenclature for a specific chain (A) in a tetramer (0) is 0A. Symmetry-related interfaces are boxed and expanded, with individual residues labeled and depicted in stick representation. Selected regions of a 2D CORD spectrum (100 ms mixing time) showing intratetramer correlations (magenta) and intertetramer correlations (green) (a and b, bottom panels). (c) Left: representative strips of the 2D CORD (top strips, 25 ms mixing time, gray; 100 ms mixing time, red), 2D NCACX (middle strip), 2D NCOCX (bottom strip), and 2D (H)NH HETCOR (right strips) spectra illustrating the sequential assignment for T77−G78. Resonances for two conformers, a and b, of T77 are indicated by dotted and solid lines, respectively. Right: interchain contacts of T77 for each chain are colored yellow. The number of scans and the number points in the direct and indirect dimensions are as follows: 2D CORD (25 ms mixing time) - 192 scans, 2048 t2 points, 840 t1 points; 2D CORD (100 ms mixing time) - 96 scans, 3072 t2 points, 96 t1 points; 2D NCACX - 2048 scans, 2048 t2 points, 96 t1 points; 2D NCOCX - 1536 scans, 3072 t2 points, 96 t1 points; and 2D (H)NH HETCOR - 80 scans, 3072 t2 points, 400 t1 points.

**Figure 5.** Selected amino acids that exhibit multiple backbone amide resonances in the 2D CORD spectra of crystalline SARS-CoV-2 NNTD. (a, b) Individual single cross-peaks reporting on a unique environment (a, pink labels) and doubled cross-peaks reporting on different environments for the different chains (b, gray labels) with their corresponding 1D 15N slices. (c) The location of amino acids (pink) possessing single amide backbone cross-peaks mapped onto the structure of chain A in the X-ray structure.
Notable is the complementarity of information obtained by MAS NMR and X-ray diffraction. In the X-ray structure, the atomic level details for individual chains and information on the quaternary arrangement in the crystal are obtained, while the strength of MAS NMR lies in providing information about dynamically disordered regions, proton positions, protonation and tautomeric states, and contacts with water molecules. To understand the dynamics of the different regions of NNTD in solution and in the crystalline forms and their role in RNA binding, it will be interesting in future studies to perform measurements of relaxation rates, chemical shifts, and dipolar anisotropy tensors.

**CONCLUSIONS**

We have determined the structure of SARS-CoV-2 NNTD by integrating MAS NMR and X-ray diffraction. Our combined approach provided atomic details of packing interfaces as well as information about disordered residues at the N- and C-termini and the functionally important RNA binding, β-hairpin loop. In addition, 1H-detected experiments at an MAS frequency of 100 kHz permitted the assignment of side-chain proton chemical shifts not available by other means. The present structure offers guidance for designing therapeutic interventions against the SARS-CoV-2 infection.

**MATERIALS AND METHODS**

**Expression and Purification of NNTD.** The recombinant plasmid for expressing SARS-CoV-2 NNTD (residues 40–174, current construct residue numbering 2–136) was prepared from GenScript based on the sequence previously reported for NNTD<sup>19</sup> and E.coli codon-optimized. The template coding for SARS-CoV-2 NNTD was subcloned into a pET28a(+) vector fused with an N-terminal hexahistidine tag (His<sub>6</sub>), followed by a tobacco etch virus (TEV) protease cleavage site, His<sub>6</sub>-TEV-NNTD<sup>19</sup>. For the expression of U-<sup>13</sup>C,<sup>15</sup>N-NNTD and U-<sup>13</sup>N-NNTD, transformed E.coli BL21...
(DE3) cells were cultured in 5 mL of Luria-Bertani (LB) medium containing 100 µg/mL of kanamycin. LB preculture was incubated at 37 °C with agitation until the OD$_{600}$ reached 1.0−1.2. Fifty milliliters of M9 medium, supplemented with 1 g/L $^{15}$NH$_4$Cl (U−$^{15}$N) or 1 g/L $^{15}$NH$_4$Cl and 2 g/L $^{13}$C$_6$-glucose (U−$^{13}$C,$^{15}$N−$^{15}$N), was inoculated with 1 mL of the LB preculture and incubated overnight at 37 °C. Following the overnight growth, 50 mL of M9 medium was transferred to 1 L of the isotopically labeled M9 medium and incubated at 37 °C. Cells were grown to an OD$_{600}$ of 1.0 and induced with a final isopropylthio-β-D-galactoside (IPTG) concentration of 0.4 mM. Following induction, cultures were harvested by centrifugation at 4000 $\mu$g/mL for solution NMR and 30 mg/mL for crystallization. The cell pellet was resuspended in lysis buffer (20 mM tris $\cdot$ HCl, 150 mM NaCl, pH 6.0) was mixed with 200 µL of 20% PEG4000, pH 6.0 at 100 K. All data were indexed and integrated using the program XDS38 and scaled using the program AIMLESS from the CCP4 suite.39 The structure was solved by molecular replacement (MOLREP, CCP4 suite) using one monomer of PDB-ID 6M3M. Structure refinement was carried out in Phenix40 with manual building in COOT41 (PDB-ID 7UW3, this work).

**Solution NMR Spectroscopy.** A 2D $^{1}$H−$^{15}$N HSQC spectrum of 850 mM U−$^{15}$N−$^{15}$N in 20 mM tris−HCl, 150 mM NaCl, 90:10 H$_2$O/D$_2$O buffer (pH 8.0) was recorded at 25 °C on a 14.1 T Bruker Neo spectrometer equipped with a triple-resonance inverse detection (TXI) probe. The Larmor frequencies were 600.13 MHz ($^{1}$H), 150.9 MHz ($^{13}$C), and 60.8 MHz ($^{15}$N). Backbone and side-chain $^{1}$H and $^{15}$N chemical shift assignments (Figure S10 and Table S5 of the Supporting Information) were obtained by comparison with SARS-CoV-2 NTD (BMRB:34511) and SARS-CoV-1 N-NTD (BMRB:6372) chemical shifts in the BMRB.29,30 $^{15}$N−$^{15}$N HMBC spectra were recorded at pH 6.3 to match the crystallization pH, with delays set to 5, 25, and 50 ms, corresponding to 1/2 of $^{1}$J and $^{2}$J coupling constants of 92, and 10 and 20 Hz, respectively.

**MAS NMR Spectroscopy.** MAS NMR spectra of U−$^{15}$N−$^{15}$N−$^{15}$N protein crystals were recorded on a 14.1 T Bruker AVIII spectrometer outfitted with a 1.3 mm HCN probe. The Larmor frequencies were 599.85 MHz (H), 150.85 MHz ($^{13}$C), and 60.75 MHz ($^{15}$N). The MAS frequencies were controlled to within ±10 Hz by a Bruker MAS controller. The actual sample temperature was maintained at ~25 °C throughout the experiment.

**Typical 90° pulse lengths were 1.3−1.5 µs for $^{1}$H, 2.6−2.9 µs for $^{13}$C, and 3.2−3.5 µs for $^{15}$N. $^{1}$H−$^{13}$C and $^{1}$H−$^{15}$N cross-polarizations were performed with an 80−100% linear amplitude ramp on $^{1}$H, with contact times of 1−1.5 and 2−2.5 ms, respectively.**
ramp was matched to the Hartmann–Hahn condition at the first spinning sideband. 2D \(^{13}\text{C}−^{13}\text{C}\) CORD,\(^{29}\) 2D NCACX, and 2D NOCCX spectra were recorded at a MAS frequency of 14 kHz. CORD mixing times were 25, 100, 250, and 500 ms, and the \(^{1}H\) radio frequency (rf) field strength during CORD mixing was 14 kHz. Band-selective \(^{13}\text{N}−^{13}\text{C}\) spectrally induced filtering in combination with cross-polarization (SPECIFIC-CP)\(^{32}\) with a contact time of 6.0−7.5 ms. SPINAL-64\(^{45}\) decoupling (90−100 kHz) was used during the evolution and acquisition periods.

2D \(^{13}\text{C}−^{13}\text{C}\) RFDPR, \(^{1}H\)-detected 2D (H)NH, and (H)CH HETCOR, as well as 3D (H)CANH and (H)CONH spectra, were recorded at an MAS frequency of 60 kHz with a 2.4 ms RFDPR mixing time. Swept-low power TPPM (15 kHz, STTPPM)\(^{36}\) was used for \(^{1}H\)-heteronuclear decoupling during acquisition. WALTZ-16\(^{16}\) broad-band decoupling was used for \(^{13}\text{C}\) and \(^{15}\text{N}\) decoupling during \(^{1}H\) acquisition. For 3D \(^{1}H\)-detected (H)CANH and (H)CONH spectra, CA-N and CO-N CP contact times were 6−7.5 ms with a contact-ampitude spin lock of about 25 kHz on \(^{13}\text{C}\) and a tangent-modulated amplitude spin lock of the mean rf field amplitude of about 35 kHz on \(^{15}\text{N}\).\(^{46}\)

Additional MAS NMR spectra were recorded on a 20.0 T Bruker AVIII spectrometer outfitted with a 0.7 mm HCND and a 1.3 mm HCN probe. The Larmor frequencies were 850.4 MHz (\(^{1}H\)), 213.9 MHz (\(^{13}\text{C}\)), and 86.2 MHz (\(^{15}\text{N}\)). The MAS frequency was 100 kHz, controlled to within ±50 Hz by a Bruker MAS controller. The sample temperature was maintained at ~25 °C throughout the experiments. Pulse lengths (90°) were 1.3 μs for \(^{1}H\), 3.15 μs for \(^{13}\text{C}\), and 3 μs for \(^{15}\text{N}\). The (H)NH spectrum was recorded using a back CP (HN) of an 800 μs contact time with an 80−100% linear amplitude ramp on \(^{1}H\); the rf field strengths were 145 kHz for \(^{1}H\) and 48 kHz for \(^{15}\text{N}\). The forward CP (NH) used a 200 μs contact time, with an 80−100% linear amplitude ramp on \(^{1}H\); the rf field strengths were 134 kHz for \(^{1}H\) and 48 kHz for \(^{15}\text{N}\). For (H)CH and (H)CCH experiments, the \(^{13}\text{C}\) CP rf field strength was set to 30 kHz; for forward and back CP, linear amplitude ramps on \(^{1}H\) were 80−100 and 100−80%; the \(^{1}H\) rf field strengths were set to 138 and 132 kHz; the contact times were 600 and 175 μs, respectively. The CC RFDPR mixing time was 0.56 ms. For all spectra, the \(^{1}H\) rf field strengths for water suppression and proton decoupling were set at 1/4 ν\(_{LH}\). A WALTZ sequence at 10 kHz was used for heteronuclear decoupling of both \(^{13}\text{C}\) and \(^{15}\text{N}\).

An additional 2D (H)NH spectrum was recorded at 60 kHz MAS, using a 1.3 mm HCN probe. The CP contact time was 4 ms, and the remainder of all conditions were identical to those at 14.1 T (see above).

Data Processing. All MAS NMR data were processed using Bruker TopSpin and NMRPipe.\(^{47}\) \(^{1}H\) resonances are referenced with respect to water at 4.7 ppm and \(^{13}\text{C}\) and \(^{15}\text{N}\) to the external standards adamantane and ammonium chloride, respectively. All 2D and 3D data sets were processed by applying 30, 45, 60, and 90° shifted sine bell apodization, followed by a Lorentzian-to-Gaussian transformation in all dimensions. Forward linear prediction to twice the number of original data points was applied in the indirect dimension for some 3D data sets, followed by zero filling. 2D and 3D \(^{1}H\)-detected data sets were processed with Gaussian and/or square sine window apodization and quadrature baseline correction.

Resonance Assignments. Spectra were analyzed using CCPN\(^{48}\) and Sparky,\(^{49,50}\) and MAS NMR backbone and side-chain \(^{1}H−^{1}N\) resonance assignments were initially carried out by comparison with solution NMR chemical shifts\(^{22−23}\) and verified by de novo backbone assignment based on 2D \(^{13}\text{C}−^{13}\text{C}\) CORD (25 ms mixing time) and RFDPR spectra, combined with 2D NCACX (25 ms mixing time), 2D NOCCX (25 ms mixing time), \(^{1}H\)-detected 2D (H)NH HETCOR, 3D (H)CANH, and 3D (H)CONH spectra. Side-chain carbon and nitrogen resonances were assigned using 2D CORD, 2D NCACX, 2D NOCCX, and 2D (H)NH spectra, and side-chain and backbone protons were assigned using \(^{1}H\)-detected 2D (H)NH, (H)CH HETCOR, and 3D (H)CCH experiments.

Structure Calculation of SARS-CoV-2 N\(^{\text{NDT}}\). The MAS NMR structure of a single N\(^{\text{NDT}}\) chain was calculated in Xplor-NIH version 2.5.5–51 using \(^{13}\text{C}−^{13}\text{C}\), \(^{15}\text{N}−^{15}\text{C}\), and \(^{1}H−^{1}N\) distance restraints, extracted from 2D CORD (100, 250, and 500 ms mixing times), NCACX, NOCCX, and (H)NH HETCOR spectra and backbone dihedral angles predicted by TALOS–N\(^{54}\) from the experimental \(^{1}H\), \(^{13}\text{C}\), and \(^{15}\text{N}\) chemical shifts. The bounds for the distance restraints were set to 1.5−6.5 Å (4.0 ± 2.5 Å) and 2.0−7.2 Å (4.6 ± 2.6 Å) for intra- and inter-residue restraints, respectively, consistent with our previous studies.\(^{50,55}\)

Calculations were seeded using the primary sequence as extended strands. One thousand structures were generated with molecular dynamics simulated annealing in torsion angle space with two successive annealing schedules and a final gradient minimization in Cartesian space, essentially as described previously\(^{55,56}\) and detailed below.

Two successive annealing schedules were used, the first in vacuum with the REPEL module and the second with an implicit solvent refinement using the EEFx module.\(^{56}\) The 10 lowest-energy structures were selected and served as input for the second schedule, and the 10 lowest-energy structures were selected and served as input for the final ensemble (PDB: 7SD4). Standard terms for bond lengths, bond angles, and improper pairs were applied to enforce the correct covalent geometry.

The first annealing calculation was essentially identical to that reported previously\(^{55,56}\), with initial random velocities at 3500 K constant-temperature molecular dynamics run for the shorter of 800 ps or 8000 steps, with the time step size allowed to float to maintain constant energy. Subsequently, simulated annealing calculations at reduced temperatures in steps of 25−100 K were carried out for the shorter of 0.4 ps or 200 steps. Force constants for distance restraints were ramped from 10 to 50 kcal/(mol Å\(^2\)). Dihedral angle restraints were disabled for high-temperature dynamics at 3500 K and subsequently applied with a force constant of 200 kcal/(mol rad\(^2\)). The force constant for the radius of gyration was geometrically scaled from 0.002 to 1, and a hydrogen bond term, HBpot, was used to improve hydrogen bond geometries.\(^{57}\) After simulated annealing, structures were minimized using a Powell energy minimization scheme.

For the second schedule performed in implicit solvent, all parameters were set as in the example EEFx of Xplor-NIH. Annealing was performed at 3500 K for 15 ps or 15,000 steps, whichever was completed first. The starting time step was 1 fs and was self-adjusted in subsequent steps to ensure conservation of energy. Random initial velocities were assigned about a Maxwell distribution at the starting temperature of 3500 K. Subsequently, the temperatures were reduced to 25 K in steps of 12.5 K. At each temperature, 0.4 ps dynamics were run with an initial time step of 1 fs. Force constants for distance restraints were ramped from 2 to 30 kcal/(mol Å\(^2\)). The dihedral restraint force constants were set to 10 kcal/(mol rad\(^2\)) for high-temperature dynamics at 3,000 K and 200 kcal/(mol rad\(^2\)) during cooling. After the EEFx module, structures were minimized using a Powell energy minimization scheme.

Structure Analysis and Visualization. Atomic rmsd values were calculated using routines in Xplor-NIH (version 2.5.3).\(^{51–55}\) The visualization of structural ensembles was rendered in PyMOL,\(^{58}\) using in-house shell/bash scripts. Secondary structure elements were classified according to STRIDE\(^{59}\) and manual inspection.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c03320.

Summary of resonance assignment of SARS-CoV-2 N\(^{\text{NDT}}\); X-ray crystal structure; 10 lowest-energy conformers of MAS NMR structures; all and selected side-chain conformations of MAS NMR and X-ray structures; multiple resonances for AS2 in the MAS NMR spectra; histidine protonation state in solution NMR; mass spectrum, crystallization, and purification of N\(^{\text{NDT}}\); 2D

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\(^1\)H–\(^1\)N HSQC NMR spectrum of \(^1\)H–\(^1\)N–\(^{\text{NNTD}}\)N, summary of all NMR experiments; MAS NMR and solution NMR chemical shifts of \(^{\text{NNTD}}\); rmsd values between X-ray and MAS NMR structures; intra- and intertetramer contacts in \(^{\text{NNTD}}\) crystals; and author contributions (PDF)

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S.S. and B.R. contributed equally to this work.

### Notes

The authors declare no competing financial interest.

The MAS NMR atomic structure coordinates and X-ray structure coordinates of SARS-CoV-2 \(^{\text{NNTD}}\) have been deposited in the Protein Data Bank under accession codes 7SD4 and 7UW3, respectively. MAS NMR chemical shifts of SARS-CoV-2 \(^{\text{NNTD}}\) have been deposited in the Biological Magnetic Resonance Data Bank under accession code 30955.

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