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Dynamic Nuclear Polarization Enhanced MAS NMR for Structural Analysis of HIV-1 Protein Assemblies

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Abstract

Mature infectious HIV-1 virions contain conical capsids comprised of CA protein, generated by the proteolytic cleavage cascade of the Gag polyprotein, termed maturation. The mechanism of capsid core formation through the maturation process remains poorly understood. We present DNP-enhanced MAS NMR studies of tubular assemblies of CA and Gag CA-SP1 maturation intermediate and report 20 – 64 fold sensitivity enhancements due to DNP at 14.1 T. These sensitivity enhancements enabled direct observation of spacer peptide 1 (SP1) resonances in CA-SP1 by dipolar based correlation experiments, unequivocally indicating that the SP1 peptide is unstructured in assembled CA-SP1 at cryogenic temperatures, corroborating our earlier results. Furthermore, the dependence of DNP enhancements and spectral resolution on magnetic field strength (9.4 – 18.8 T) and temperature (109 – 180 K) was investigated. Our results suggest that DNP-based measurements could potentially provide residue-specific dynamics information by allowing for the extraction of temperature dependence of the anisotropic tensorial or relaxation parameters. With DNP, we were able to detect multiple well-resolved isoleucine sidechain

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Supporting Information

Amino acids that exhibit loss of NMR signal due to the presence of AMUPol at room temperature, chemical shift input values used as input for PLUQ secondary structure prediction, 9.4 T and 18.8 T ¹³C-¹³C DNP-enhanced correlation spectra of CA-SP1-NC at 100 and 180 K, the primary amino acid sequence of CA-SP1-NC (HXB2 strain) protein and, complete citation for references ⁹, ¹⁵, ¹⁶, ⁴⁸ and ⁶⁸. This information can be found on the internet at <http://pubs.acs.org>.

conformers, unique intermolecular correlations across two CA molecules, and functionally relevant conformationally disordered states such as the 14-residue SP1 peptide, none of which are visible at ambient temperatures. The detection of isolated conformers and intermolecular correlations can provide crucial constraints for structure determination of these assemblies. Overall, our results establish DNP-based MAS NMR as an excellent tool for characterization of HIV-1 assemblies.

Keywords

magic angle spinning; MAS; dynamic nuclear polarization; DNP; HIV-1 capsid protein

Introduction

In human immunodeficiency virus (HIV-1), mature virions contain conical capsids comprised of multiple copies of the CA capsid protein, generated by the proteolytic cleavage cascade of the Gag polyprotein, termed maturation.^{1,2} The conical capsid encloses two copies of viral RNA and several ancillary proteins necessary for the HIV-1 replication.³⁻⁴ Despite numerous studies into virus maturation by multiple techniques, its mechanism remains poorly understood, particularly at atomic resolution. Gag is comprised of several domains: capsid (CA), nucleocapsid (NC), matrix (MA), and p6, as well as spacer peptides 1 and 2 (SP1 and SP2, respectively), as shown in Figure 1a. Gag cleavage during maturation proceeds sequentially, resulting in several maturation intermediates. The condensation of CA protein into conical cores occurs at the final maturation step, following cleavage of the 14-residue SP1 peptide from the C-terminal domain of the CA protein (see Figure 1b).³ While the cleavage of the SP1 peptide is accepted to be the trigger of the capsid condensation,^{3, 5, 7} the mechanism by which this process occurs, i.e., through remodeling of the immature lattice or *de novo* lattice formation, remains a subject of debate.^{8, 10} Integral to this controversy in the field is the question of the SP1 peptide conformation in the Gag polyprotein and in the CA-SP1 maturation intermediate. Our recent work has shown that at temperatures above 0 °C, SP1 is dynamic and unstructured in assembled CA-SP1, indirectly supporting the hypothesis that capsid condensation occurs *de novo* rather than through gradual lattice remodeling.⁹ However, direct evidence in support of this hypothesis has not yet been available. Previous studies by cryo-EM microscopy (cryo-EM), cryo-electron tomography (cryo-ET) and solution NMR have investigated the conformation of the SP1 peptide in the context of CA-SP1-NC, Gag polyprotein and immature virus like particles (VLPs) assembled from the Gag lacking a portion of MA domain. Cryo-EM studies on Gag and immature VLPs suggest that the SP1 peptide adopts a helical structure.^{5, 11, 12} In contrast, solution NMR studies on unassembled Gag found that the SP1 peptide has a random coil conformation.^{13, 14} To the best of our knowledge, assemblies of the CA-SP1 maturation intermediate, lacking the MA and NC domain, have not been investigated by cryo-EM yet. Atomic-level structures and details of the maturation process, including the structures of Gag maturation intermediates constitute a prerequisite for designing small-molecule maturation inhibitors, currently a topic of intense interest in the HIV research community.

Structural characterization of the HIV-1 CA capsid has been performed at various levels of resolution by cryo-electron tomography cryo-ET,¹⁵ cryo-EM,^{15,19} X-ray crystallography,^{20,27} and solution NMR spectroscopy,^{16, 28,29} and the structural organization of the capsid is relatively well understood. Cryo-ET studies of native HIV-1 cores revealed heterogeneity in the conical structure.¹⁵ Cores are pleiomorphic with ca. 1,200 copies of CA protein forming ~216 hexameric and ~12 pentameric units that condense into a closed ovoid.^{15, 19} Despite the availability of an all-atom model of the capsid by hybrid cryo-ET, molecular dynamics and solution NMR approaches,¹⁵ a direct determination of the atomic-resolution structure of the full assembly has not been performed.

Magic angle spinning (MAS) NMR spectroscopy is uniquely suited for investigations of HIV-1 protein assemblies at atomic resolution, as shown by us and by others.^{9, 30,34} While MAS NMR experiments have provided important insights into the structure and dynamics of HIV-1 assemblies, they often suffer from limited sensitivity, precluding detection of low-concentration moieties of functional and structural importance, such as minority species (e.g., pentameric units of the CA protein in the conical capsids in the presence of a majority of hexamers). Furthermore, detection of mobile species whose resonances are broadened and/or weakened due to the presence of dynamics, is a challenge, as we have seen in our prior studies.^{9, 31} These issues will likely be exacerbated when investigating larger HIV-1 protein assemblies, such as virus like particles (VLP) formed by the Gag polyprotein.

Dynamic nuclear polarization (DNP) is an emerging technique that provides very large sensitivity enhancements (with a ~660 fold theoretical limit for protons), making it a promising tool to study low-concentration sites in the context of macromolecular assemblies, such as HIV-1 assemblies (e.g., pentameric declinations in the conical capsid, see above).^{35,36} In this report, we present a DNP-based investigation of several assemblies of HIV-1 proteins: the final Gag cleavage product CA, the maturation intermediate CASP1, and the Gag construct CA-SP1-NC that lacks MA, SP, and p6 domains.

20-64-fold sensitivity enhancements are observed at 14.1 T for CA and CA-SP1 assemblies, using AMUPol as the paramagnetic biradical.³⁷ In these conditions, we were able to directly observe functionally important residues that are invisible in room-temperature dipolar-based MAS NMR, cryo-EM, and X-ray measurements due to dynamic disorder. Specifically, we have detected and assigned the majority of resonances of the SP1 peptide residues in the CA-SP1 maturation intermediate and corroborated that the peptide remains unstructured at cryogenic temperatures. We also show that at 14.1 T, the resolution of 2D homonuclear correlation DNP-MAS spectra is excellent, permitting site-specific resonance assignments for multiple cross peaks, in particular for aromatic sidechains. Additionally, the DNP spectra revealed the presence of multiple sidechain conformers for several residues as well as unique correlations that were not visible at room temperature, such as those arising from an intermolecular interaction between Y145 and R162, residues comprising the CTD-CTD interface in the capsid.

For these experiments, a novel sample preparation protocol is proposed, to preserve the tubular morphology of the otherwise fragile assemblies during freeze-thaw cycling and addition of cryo-protectant. The effects of the magnetic field (9.4 – 18.8 T) and temperature

(109 - 180 K) on the resolution and sensitivity of the DNP-enhanced spectra were also investigated. Notably, DNP enhancements revealed that although the sensitivity gain was attenuated by 3-fold at 180 K, no observable difference in resolution was detected as a function of temperature. Our results show, however, that variable temperature DNP measurements may provide residue-specific dynamic information.

To our knowledge, our work is the first DNP-enhanced MAS NMR study of multi-copy protein assemblies. Taken together, our results indicate that DNP based MAS NMR is an excellent tool for structural characterization of HIV-1 protein assemblies and yields unique structural information, not accessible through other techniques, including cryo-EM, X-ray crystallography, and room-temperature NMR.

Materials and Methods

Sample Preparation

Expression and purification of CA, CA-SP1, and CA-SP1-NC (HXB2 strain) were performed as reported previously.^{9, 38} Tubular assemblies of CA and CA-SP1 were prepared from 32 mg/mL protein solutions in 25 mM phosphate buffer (pH 5.5) containing 2.4 M NaCl, according to the published procedure.⁹ The solutions were incubated at 37° C for 1 h and stored at 4° C for subsequent experiments. For MAS NMR experiments at 19.96 T, the samples were packed in 3.2 mm thin wall MAS NMR rotors.

For DNP experiments at 14.1 T, tubular assemblies of CA and CA-SP1 were prepared following the general protocol above, in buffer containing 90% D₂O: 10% H₂O. The biradical, AMUPol (15-[(7-oxyl-3, 11-dioxa-7-azadispiro [5.1.5.3] hexadec-15-yl) carbamoyl][2-(2,5,8,11-tetraoxatridecan-13-ylamino)}-[3,11-dioxa-7-azadispiro [5.1.5.3] hexadec-7-yl]) oxidanyl)³⁷, was added to the sample to the final concentration of 8 mM, and gently stirred. AMUPol dissolved immediately with no visible, undissolved particles in the sample, and the color of the tubular assemblies turned light orange. Excess supernatant was then removed from the precipitate, followed by the addition of 20% (v/v) d8-glycerol on top, without disturbing the pellet, and the sample was incubated overnight at 4° C. The excess glycerol solution was removed after an overnight incubation, and samples were packed in 3.2 mm sapphire DNP MAS NMR rotors.

For DNP experiments at 18.8 T, tubular assemblies of CA were prepared using the same protocol as described above, except that TOTAPOL³⁹ was used as the biradical. This sample gave an enhancement of 4 at 18.8 T. TOTAPOL was then removed by buffer exchange into a buffer containing 25 mM phosphate (pH 5.5) and 2.4 M NaCl. Upon buffer exchange, AMUPol was added to the sample. No significant changes in the enhancement factors for DNP experiments were observed after removal of TOTAPOL and addition of AMUPol. The assemblies of CA-SP1-NC, comprised of a mixture of tubes and cones, were prepared by incubating a mixture of (TG)₅₀ DNA (in 25mM NaPi, pH 5.5 90% D₂O/10% H₂O, 20% d8-glycerol) and CA-SP1-NC (in 25mM NaPi pH 5.5 90% D₂O/10% H₂O, 20% d8-glycerol) until the final concentrations of TG50 and CA-SP1-NC protein were 60 μ M and 10 mg/ml, respectively. Upon formation of the precipitate of CA-SP1-NC assemblies, AMUPol was

added to the final concentration of 8 mM. The sample was stirred gently and stored at 4° C for subsequent experiments.

For DNP experiments at 9.4 T, CA-SP1-NC sample (in 2.4M NaPi) was prepared by incubating the protein preparation in cryoprotectant solution to give 1:3 v/v of protein: solution. The cryo-protectant solution was 50% v/v 75% D₂O/25% H₂O, and 50% v/v d8-glycerol, with a TOTAPOL concentration needed to give a 10 mM final concentration. A control sample containing no TOTAPOL was examined as well.

Transmission Electron Microscopy

The sample morphologies were characterized by TEM analysis, performed in a Zeiss CEM 902 transmission electron microscope operating at 80 kV. Samples were stained with ammonium molybdate (5% w/v), deposited onto 400 mesh, formval/carbon-coated copper grids, and dried for 40 min. Some of the assemblies were analyzed using a Zeiss Libra 120 transmission electron microscope operating at 120 kV. Samples were stained with uranyl acetate (5% w/v), deposited onto 400 mesh, formval/carbon-coated copper grids, and dried for 40 min in the air. The copper grids were pretreated with Pelco easiGlow Discharge Unit to deposit a charge, so that the assemblies were uniformly spread on the grid surface and adhere to it.

NMR Spectroscopy

MAS NMR spectra of tubular assemblies of CA and CA-SP1 were acquired on a Bruker 19.96 T AVIII spectrometer outfitted with a 3.2 mm E^{free} CPMAS HCN probe. The Larmor frequencies were 850.400 MHz (¹H) and 213.855 MHz (¹³C). The spectra were collected at the MAS frequency of 14.000 kHz, and the temperature was maintained at 277 ± 0.5 K throughout the experiments. The typical pulse lengths were 2.9 μs for ¹H, 4.2 μs for ¹³C. ¹H – ¹³C cross-polarization (CP) was performed with a linear amplitude ramp (80–100%); the CP contact time was 2 ms. SPINAL-64 decoupling (80 kHz) was applied during the evolution and acquisition periods. The 2D ¹³C-¹³C correlation spectra were acquired as DARR^{40,41}, PDSD⁴² or CORD⁴³ experiments at 14.1, 18.8 and 19.96 T, respectively. The ¹H field strength during DARR was 14 kHz, and the DARR mixing time was 50 ms.

DNP-enhanced MAS NMR spectra of tubular assemblies of CA and CA-SP1 were acquired at 14.1 T at Bruker Biospin on a Bruker AVIII spectrometer equipped with a 3.2-mm triple-resonance low temperature DNP MAS probe. The Larmor frequencies were 600.080 MHz (¹H) and 150.905 MHz (¹³C), and the microwave frequency was 395.18 GHz. MW irradiation was generated by a second-harmonic gyrotron providing 12 W of microwave power at the sample. The sample temperature was calibrated using KBr.⁴⁴ The typical pulse lengths were 2.5 μs (¹H), 4 μs (¹³C), and the ¹H-¹³C CP contact time was 2 ms. The MAS frequency was 12.5 kHz, and DARR mixing time was 40 ms.

DNP-enhanced MAS NMR measurements at 18.8 T on tubular assemblies of CASP1-NC were acquired on a Bruker AVIII spectrometer equipped with a 3.2-mm triple-resonance low temperature MAS probe. The Larmor frequencies were 799.723 MHz (¹H) and 295.98 MHz (¹³C), and the microwave frequency was 527 GHz. MW irradiation was generated by a second-harmonic gyrotron. Data were recorded at a temperature of 110 K. The sample

temperature was calibrated using KBr. The typical pulse lengths were 2.5 μ s (^1H), 4.75 μ s (^{13}C), and the ^1H - ^{13}C CP contact time was 2 ms. The MAS frequency was 10 kHz, and the PDSD mixing time was 50 ms.

The 9.4 T DNP-enhanced MAS NMR spectra of frozen solutions of CA-SP1-NC were acquired on a Bruker AVIII spectrometer equipped with a 3.2-mm triple-resonance low temperature DNP-MAS probe. The Larmor frequencies were 400.130 MHz (^1H) and 100.622 MHz (^{13}C), and the microwave frequency was 263 GHz. MW irradiation was generated by a first-harmonic gyrotron. Data were recorded at temperatures of 110 and 182 K. The typical pulse lengths were 2.6 μ s (^1H), 3.3 μ s (^{13}C), and the ^1H - ^{13}C CP contact time was 0.9 ms. The MAS frequency was 8.889 kHz, and the PDSD mixing time was 10 ms.

Processing and Analysis of NMR spectra

All MAS NMR spectra were processed with NMRpipe.⁴⁵ Forward linear prediction to twice the number of the original data points was used in the indirect dimension in some data sets followed by zero filling to twice the total number of points. 60° or 30° shifted sine bell apodization followed by a Lorentzian-to-Gaussian transformation was used in all dimensions. All DNP-enhanced MAS NMR spectra were processed in TopSpin using Gaussian apodization in the direct dimension and, for some spectra, sine bell square apodization was applied in the indirect dimension. All data sets were analyzed using Sparky.⁴⁶ The effective DNP enhancements were calculated based on relative signal ratios in 1D ^{13}C CP MAS spectra acquired with and without microwaves. The secondary structure analysis based on chemical shifts was done by PLUQ.⁴⁷ The chemical shift input values used for PLUQ are provided in Table S2. The predictions were based on the highest probability secondary structure for the assigned amino acid. It is noteworthy that, for a given set of resonances, PLUQ also provides statistics for other likely spin-systems and their corresponding secondary structures. Such predictions for spin-systems different from the assigned amino acid were ignored.

Results and Discussion

DNP Enhanced MAS NMR of HIV-1 Capsid Assemblies: Sensitivity and Resolution

Effect of Biradical and Freeze-thaw Cycling—Based on our TEM study, we realized that CA and CA-SP1 tubular assemblies are generally destroyed when frozen⁹ or upon mixing with cryoprotectants, such as glycerol. Therefore, we have developed a new sample preparation protocol (see Materials and Methods) for the incorporation of DNP biradicals and cryoprotectants whereby the cryoprotectant is added on top of the assemblies without any stirring, such that the morphology remains intact in their presence and the assemblies are not destroyed upon temperature cycling. As demonstrated by the TEM images in Figure 2, using this sample preparation protocol, the tubular structure of CA assemblies is preserved during several freeze-thaw cycles. The effect of biradical (AMUPol) addition and temperature cycling on CA assemblies was monitored by recording 2D CORD (COmbined R2_n^v-Driven)⁴³ spectra at 277 K, acquired on samples prepared without AMUPol and with AMUPol before and after DNP experiments. As illustrated in Figure 2, signals in all three data sets overlay remarkably well, indicating that the morphology of the tubular assemblies

remains intact. A slight broadening of some of the resonances is observed for the sample containing AMUPol; this apparent broadening is not uniform and signal loss for less than 5% of amino acids at 277 K occurs; resonances associated with some of these residues reappear in the DNP-assisted measurements (summarized in Table S1 of the Supporting Information). This finding suggests that signal broadenings or intensity losses are associated with the paramagnetic effects caused by the presence of the biradical.

DNP Enhancements: Magnetic Field Dependence—Enhancements and resolution in DNP experiments were examined at three magnetic field strengths ($B_0 = 9.4$ T, 14.1 T and 18.8 T) using three different sample preparation protocols, namely i) mixing in the biradical/glycerol into CA-SP1-NC assemblies and freezing the solution (for 9.4 T studies), ii) adding biradical/glycerol to the tubular CA and CA-SP1 assemblies without mixing to preserve the tubular structure (for 14.1 T studies), and iii) assembling CA-SP1-NC from solutions containing biradical/glycerol by adding (TG)₅₀ DNA. Table 1 summarizes the enhancements observed for various sample preparations as a function of field strength and temperature.

The measurements at 9.4 T and temperatures of 110 and 182 K were performed on a frozen solution of CA-SP1-NC protein mixed with DNP reagents. These experiments were our first attempt at investigating HIV-1 capsid assemblies by DNP-enhanced MAS NMR, and the sample preparation protocol was not optimized. Specifically, sample conditions were such that CA did not assemble into tubes. Nevertheless, these experiments yielded 20- and 2- fold sensitivity enhancements at temperatures of 100 and 180 K, respectively. Based on chemical shift comparisons we recognized that CA-SP1-NC is well folded. The spectra are presented in Figure S1 of the Supporting Information. With optimized sample preparation conditions these enhancements are expected to be significantly higher, as we observed at 14.1 T.

To preserve the integrity of the tubular morphology, samples of CA and CA-SP1 assemblies were prepared by the addition of biradical and glycerol to preassembled tubes for DNP measurements at 14.1 T without mixing, as detailed in the Materials and Methods section. With this protocol, we observed 64- and 20- fold enhancements at 109 K and 180 K, respectively (Figure 2). Only 3 hours of signal averaging were required to collect a 2D ¹³C-¹³C DARR spectrum for this sample at low temperature. For tubular assemblies of CA-SP1 we observed 20-fold sensitivity enhancements at 14.1 T and 109 K, and the 2D ¹³C-¹³C DARR spectrum took 12 hours to acquire. The DNP enhancements for CA-SP1 are 3-fold smaller than observed for CA assemblies at the same temperature, but still permitted the extraction of structural information unattainable at 277 K (discussed below). At present we do not understand the reason for this 3-fold difference in enhancement, however we speculate that these may be due to different densities of the tubular assemblies of CA and the CA-SP1 upon centrifugation and prior to the addition of the biradical and glycerol, or to different local concentrations of the polarizing agents. A future systematic study will be performed to address this issue.

For DNP experiments at 18.8 T, the tubular CA assemblies prepared with TOTAPOL gave 4-fold enhancements (Figure 3). Assemblies of CA-SP1-NC in the presence of (TG)₅₀ DNA were also investigated at this field. Upon the addition of DNA, CA-SP1-NC assembles into a mixture of tubes and cones (Figure 1b) that are stable and do not dissolve in the presence of

glycerol. Therefore, samples for DNP measurements were generated by adding glycerol and biradical directly to the protein solution prior to the assembly, as described in the Methods section. For samples prepared in this fashion, ca. 7-11-fold DNP enhancements were observed at 110 K and 18.8 T; a ^{13}C - ^{13}C DNP-enhanced DARR spectrum is provided in Figure S2 of the Supporting Information. These enhancements, albeit much lower than those obtained at 9.4 and 14.1 T, are comparable to those reported in literature at 18.8 T on biological assemblies⁴⁸ and still translate to an impressive reduction in experimental times (more than a factor of 16-50, without accounting for Boltzmann effects, differential T_1 relaxation and quenching factors). Even though the enhancements reported here at 9.4, 14.1 and 18.8 T are not directly comparable, as the experiments were recorded on different sample preparations, we nevertheless obtained significant amplification factors at all fields and, given that the current report is the first DNP study on CA capsid protein assemblies, we believe our current results are very encouraging, particularly at 14.1 T.

Spectral Resolution and Multiple Conformers: Temperature Effects—A

superposition of a 14.1 T DNP-enhanced DARR spectrum acquired at 109 K and a 20.0 T CORD spectrum, acquired at 277 K is provided in Figure 3a. The DNP-DARR data set was processed with either a Gaussian or a sine bell apodization; the corresponding spectra are shown in black and blue, respectively. Surprisingly, two sets of signals (narrow and broad), characterized by slow and fast transverse lifetimes, T_2^* , are present in the DNP-DARR spectrum. These two different sets of resonances can be differentiated in the spectra that are processed with different degrees of resolution enhancement. Broad signals with shorter T_2^* (Figure 3a, grey spectrum) are observed when the data sets are processed with mild resolution enhancement, whereas, a higher degree of resolution enhancement removes the intensity of the broad signal component and retains sharp signals with longer T_2^* (Figure 3a, blue spectrum). Interestingly, the spectrum with the narrow signals corresponds closely to the spectrum acquired without microwaves at 277 K, as seen in Figure 3a, indicating that the structure of the tubular assemblies is intact. The intensities of the isolated and narrow signals of the DNP-enhanced DARR spectrum at 109 K were approximately 8-fold higher than that of the spectrum acquired without microwaves at 277 K. The difference in line-widths in these sets of resonances may have either a homogeneous or an inhomogeneous origin.

In the first case, we speculate that the broad and narrow signals may originate from the CA moieties that are in close proximity (and thus subject to paramagnetic relaxation enhancement) and distal to the biradical, respectively. In this context it will be very interesting to explore in the future whether AMUPol diffuses into the inner channels of the tubes or remains on the outer surface. If the linewidth differences were indeed associated with AMUPol being inside and outside the tube, it may be possible to probe the organization of outer and inner surfaces in tubular, spherical, and conical assemblies of proteins of all kinds as was reported recently.^{48,50}

In the second case, broadening in the DNP spectra may be due to the presence of multiple sidechain conformers that are all present and not averaged at cryogenic temperatures. This has also been seen in other proteins.^{51,53} For example, different rotameric states of I104 sidechains may explain the larger linewidths of the corresponding ^{13}C resonances with respect to those of I37, as seen in the Figure 3a. Therefore, narrow signals in the DNP

spectra are useful for high-resolution structural analysis while broad signals may potentially provide information on the conformational distributions.

The DNP-assisted measurements on the CA tubular assemblies at 18.8 T yielded 4-fold sensitivity enhancements. It is noteworthy that these measurements were done using TOTAPOL biradical. These spectra displayed much higher resolution compared to those acquired at 14.1 T (Figure 3b). As shown in Figure 3b, resonance assignments can be made for various well-resolved signals based on the previous room temperature studies reported by us.³⁰ Particularly, several valine, leucine and tryptophan residues were readily identified. However, signals from threonine and serine residues were missing, and so were several inter-residue, multiple bond correlations (for example, Gly-Val) that were observed at 14.1 T (Figure 3b).

We also investigated the influence of temperature on spectral resolution. As demonstrated previously, the resolution of DNP spectra increases with temperature while the sensitivity enhancements decreases; therefore, “high-temperature” DNP has been proposed as a compromise, balancing the resolution and sensitivity gains.⁵⁴ To our surprise, we observed no resolution enhancements in DNP experiments conducted at 180 K compared to 109 K for tubular CA assemblies (Figure 3c). Given the 3-fold loss of sensitivity at 180 K compared to 109 K, it appears that for CA assemblies 109 K is the optimal temperature for structural investigations, albeit under these conditions broad, low-intensity spectral features are also present in the data processed without resolution enhancement filters. At the same time, DNP-based measurements at variable temperatures may permit the extraction of temperature-dependent anisotropic tensorial or relaxation parameters, thereby providing valuable information on residue-specific dynamics.⁵⁵⁻⁵⁸ For some residues, multiple well-resolved sidechain rotamers were present at 180 K, and the corresponding signals are missing at 109 K. This can be easily appreciated for I37 from the inset of Figure 3c. The loss of signals at 109 K might be a result of additional rotamers giving rise to multiple weak peaks that are not visible with sensitivity-enhanced processing.

Detection of Functionally Important “Invisible” Structural Regions in HIV-1 Capsid Assemblies by DNP NMR

CA-SP1 Maturation Intermediate: Detection of Dynamically Disordered Unstructured SP1 Peptide—The conformation of the SP1 peptide (14 residues) has been a subject of multiple investigations. Solution NMR studies of isolated SP1 suggested that it could adopt a helical or coil conformation depending on the concentration and solvent.⁵⁹⁻⁶⁰ We have previously deduced from MAS NMR at 277 K that in tubular CA-SP1 assemblies, SP1 is a dynamic random coil.⁹ Under those conditions no SP1 signals were detected in the dipolar correlation spectra, and only a subset of SP1 resonances was present in scalar-based INADEQUATE experiments. We expected that under the conditions of the current DNP experiments (cryogenic temperatures in conjunction with sensitivity enhancements), the SP1 resonances should be detectable in dipolar-based experiments. Indeed, as illustrated in Figure 4, SP1 resonances are clearly present in the 14.1 T DNP-DARR spectra of CA-SP1 assemblies. Based on a single DARR measurement, we were able to assign resonances from the SP1 peptide and the CTD of CA directly preceding the SP1

residues (Figure 4, Table 2). These resonances are narrow (~ 0.5 ppm) compared to other isolated and well-resolved peaks in the spectrum (1.5–2.0 ppm), indicating high conformational homogeneity. Only because of the restricted motional mobility of these residues under these conditions could these resonances be detected, which otherwise cannot be observed at ambient temperatures by dipolar-based MAS NMR or at cryogenic temperatures by cryo-EM. Secondary chemical shift analysis indicates that the SP1 peptide is a random coil conformation, while the CA residues directly preceding SP1 are helical (Table 1). Therefore, DNP MAS NMR clearly shows that the SP1 peptide in assembled CA-SP1 is in a random coil structure over the broad temperatures range from 109 to 277 K. Our current findings are in agreement with the cryo-ET results obtained on the *in vitro* assembled CA-SP capsids from Rous sarcoma virus (RSV), where the SP peptide in the virus core was found to be unstructured and hence invisible.⁸ Importantly, in contrast to cryo-ET, where electron density associated with SP1 could not be seen due to static positional disorder with respect to the CA capsid protein, SP1 is directly observed in the DNP NMR spectra acquired at similar temperatures, providing important missing information and complementary data to room-temperature NMR experiments.⁹

Detection and Assignment of Aromatic Side Chains and Dynamic Residues in CA—Detection of resonances from aromatic side chains is generally challenging in ^{13}C - ^{13}C homonuclear spectra. Even though the CORD sequence developed by us previously yielded aromatic sidechains resonance correlations,⁴³ detection of mobile aromatic sidechains resonances is not feasible at room temperature. In contrast, under DNP conditions, detection of aromatic sidechains is expected to be straightforward.⁶¹ Indeed, as shown in Figure 5, strong cross peaks belonging to the aromatic sidechains resonances are present in the DNP-DARR spectra of CA-SP1 assemblies. CA-SP1 has eighteen aromatic residues, and we were able to unequivocally assign twelve of these based on the chemical shifts reported by us previously from solution and MAS NMR studies.^{9, 30, 62}

As shown in Figure 5, some of the aromatic sidechains exhibit multiple cross peaks associated with different conformers. Such multiple conformers may be associated with molecular motions of the aromatic rings that are frozen at cryogenic temperatures. The evidence of such dynamics under non-DNP conditions has been recently reported for aromatic sidechains.^{63,67} In principle, analysis of the conformational space and the populations could provide a glimpse into the type of motions that exist. However, data at variable temperatures will be required for a quantitative analysis of such dynamics, which can depend on motional timescales, amplitudes, populations and potential energy barriers. It will be interesting to perform such analysis in the forthcoming studies.

In the DNP-DARR spectra, we also readily detected cross peaks corresponding to residues in the hinge region of CA, playing a critical role in the capsid assembly.⁶⁸ At temperatures of 253 – 277 K, these hinge region residues exhibit millisecond timescale dynamics, precluding their facile detection and assignment.³¹ For example, a key hinge residue, Y145, whose mutations render CA incapable of assembly,^{16, 68} has been difficult to detect in 2D MAS NMR experiments.³¹ In contrast, in the current DNP experiments, the Y145 peaks were readily observed and assigned, as shown in Figure 5c. Remarkably, the DNP-DARR spectra also revealed intermolecular correlations between Y145 and R142, across the CTD-

CTD interface, further validating the assignments (Figure 5c). It is noteworthy that cross peaks associated with this correlation are stronger at 180 K than at 109 K, owing to dynamics of Y145. This is the first time that intermolecular correlations involving aromatic residues could be observed for CA protein assemblies. Intermolecular correlations, such as Y145-R142, provide critical restraints for structure determination of capsid assemblies. While systematically collecting and assigning such intermolecular correlations is beyond the scope of this manuscript, major efforts in our laboratory are being devoted to such studies, aimed at deriving the atomic-level structure determination of HIV-1 CA assemblies.

Conclusions

Here we demonstrated that DNP-based MAS NMR experiments hold great promise for structural and dynamics analysis of HIV-1 CA protein assemblies. With currently attainable sensitivity enhancements as high as 64-fold, DNP measurements open the doors for analysis of functionally relevant “invisible” species, such as dynamically disordered states, which are not detectable by cryo-EM, X-ray crystallography and room temperature dipolar-based NMR spectroscopy. Tubular assemblies of CA and CA-SP1 yielded remarkably high-resolution DNP spectra at 109 K. The direct detection of SP1 peptide resonances in CA-SP1 assemblies demonstrated here, provided first hand direct evidence that the peptide is unstructured and in a random coil conformation at cryogenic temperatures. We also observed multiple conformers for several aliphatic and aromatic sidechains in the CA capsid protein. Temperature-dependent analysis of these conformers and their populations appears to be a promising future venue for detailed characterization of their dynamic behavior. Given the excellent quality of the current data, we are confident that DNP-based atomic-resolution structure determination of HIV-1 CA assemblies is feasible in the near future.

Supplementary Material

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Figure 1.

(a) Schematic representation of the domain structure of the Gag polyprotein. The arrows represent the cleavage sites in the proteolytic cleavage cascade of Gag¹. (b) The final step of the maturation process involves the cleavage of the SP1 peptide. The TEM images of tubular assemblies of CA-SP1 and CA investigated by DNP-enhanced MAS NMR spectroscopy are shown below.

Figure 2.

(a-c) An overlay of three 20.0 T ^{13}C - ^{13}C CORD spectra of tubular assemblies of HIV-1 U- ^{13}C , ^{15}N -CA without AMUPol (black), with AMUPol before DNP measurements (blue), and with AMUPol after DNP experiments (yellow). The MAS frequency was 14 kHz; the CORD mixing time was 50 ms. (d, e) Negatively stained TEM images of HIV-1 CA tubular assemblies containing 8 mM TOTAPOL and 20% (v/v) glycerol: freshly prepared (d), and after one freeze-thaw cycle (e). (f-h) ^{13}C CPMAS spectra of tubular assemblies of HIV-1 U- ^{13}C , ^{15}N -CA: (f) $B_0 = 20.0$ T, $T = 277$ K, $\nu_r = 14$ kHz; (g) $B_0 = 14.1$ T, $T = 280$ K, $\nu_r = 12.5$ kHz; and (h) $B_0 = 14.1$ T, $T = 109$ K, $\nu_r = 12.5$ kHz. (i-j) ^{13}C DNP-CPMAS spectra of tubular assemblies of HIV-1 U- ^{13}C , ^{15}N -CA recorded at 14.1 T and $\nu_r = 12.5$ kHz, (i) $T = 109$ K; (j) $T = 180$ K. All samples contained 8 mM AMUPol. The spectra shown in (g-j) were recorded on the sample that contained 20% d8-glycerol.

Figure 3.

DNP-enhanced ^{13}C - ^{13}C correlation spectra of tubular assemblies of U- ^{13}C , ^{15}N CA. (a) Acquired at 14.1 T and 109 K processed with Gaussian apodization in both dimensions (grey) and a 30-degree sine bell square apodization in the F1 dimension (blue). A CORD spectrum acquired at 277 K and 20.0 T is overlaid on top (yellow). The top insets display an expansion of the I104 and I37 sidechain resonances, indicative of multiple conformations at cryogenic temperature; the top right inset shows 1D slices of I104 C δ 1 resonance for the two spectra. (b) Expansions showing an overlay of 18.8 T (brown, PDSD) and 14.1 T (blue, DARR) spectra with well resolved signals observed at 18.8 T. (c) An overlay of 14.1 T

spectra acquired at 109 K (blue, $\varepsilon = 64$) and 180 K (black, $\varepsilon = 20$). The inset around I37 C δ 1 resonance shows signals arising from multiple conformers present at T=180 K but missing at T=109 K. The other insets represent expanded regions of the spectrum, illustrating the presence of many well-resolved signals. The spectra in (b) and (c) were processed with Gaussian and 30-degree sine bell square apodization functions in the F2 and F1 dimensions, respectively. The MAS frequency was 12.5 kHz (14.1 T) and 10 kHz (18.8 T). The mixing time was 40 ms (14.1 T) and 60 ms (18.8 T).

Figure 4.

The amino acid sequence of the SP1 peptide and an overlay of DNP-enhanced ^{13}C - ^{13}C DARR spectra at 109 K and 14.1 T of tubular assemblies of U- ^{13}C , ^{15}N CA (blue) and CA-SP1 (black). The MAS frequency was 12.5 kHz, and the mixing time was 40 ms. Assigned SP1 resonances are labeled by residue name and number.

(a-b) Aromatic correlations observed in the 14.1 T DNP-enhanced ^{13}C - ^{13}C DARR spectrum of tubular assemblies of U- ^{13}C , ^{15}N CA-SP1 at 109 K; (c) overlay of the 14.1 T DNP-enhanced ^{13}C - ^{13}C DARR spectra of tubular U- ^{13}C , ^{15}N CA assemblies at 109 K (blue) and 180 K (black), showing Y145 resonances and their correlations with R162; (d) TEM image of tubular CA assemblies containing hexameric units of CA protein as shown in (e), PDB code: 3J34 and; (f) Distance across the interface between R162 and Y145 residues from different CA monomers that give rise to the intermolecular correlations. The MAS frequency was 12.5 kHz, and the mixing time was 40 ms.

Table 1

A Summary of Signal Enhancements Observed by DNP for Different Temperatures, Magnetic Field Strengths, and Sample Preparation Protocols

Protein	Sample Preparation Protocol ^a	B ₀ (T)	Temperature	Enhancement
CA-SP1-NC	Frozen protein solution	9.4	110 K	20 ^b
CA-SP1-NC	Frozen protein solution	9.4	182 K	2 ^b
CA	Glycerol added after protein assembly	14.1	109 K	64 ^b
CA	Glycerol added after protein assembly	14.1	180 K	20 ^b
CA-SP1	Glycerol added after protein assembly	14.1	109 K	20 ^b
CA	Glycerol added after protein assembly	18.8	110 K	4 ^c
CA-SP1-NC	Glycerol premixed before protein assembly	18.8	110 K	7-11 ^b

^aFor detailed sample preparation protocols, see Material and Methods.

^bEnhancements obtained from AMUPol biradical

^cEnhancements obtained from TOTAPOL biradical.

Table 2

Secondary Structure of the SP1 Peptide and the Preceding CA Residues in Tubular Assemblies of CA-SP1.

	109 K	277 K ^a
CA residues preceding the SP1 peptide		
K227	Coil	Coil
V230/238	Sheet	Coil (NL4-3/A92E) ^b
L231	Helix	Coil
A234/242	Helix	Helix
M235/245	Helix	Coil (NL4-3/A92E) ^b
SP1 peptide residues		
T239	Coil	Coil
I244	Helix	Coil (NL4-3/A92E) ^b
A	Helix	N/A
A	Coil	N/A
M235/245	Coil	Coil (NL4-3/A92E) ^b

^aFrom ref⁹^bSignals corresponding to these residues were not detected for CA of the HXB2 strain