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Solid-State NMR Spectroscopy of Protein Complexes

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Abstract

Protein-protein interactions are vital for many biological processes. These interactions often result in the formation of protein assemblies that are large in size, insoluble and difficult to crystallize, and therefore are challenging to study by structure biology techniques, such as single crystal X-ray diffraction and solution NMR spectroscopy. Solid-state NMR (SSNMR) spectroscopy is emerging as a promising technique for studies of such protein assemblies because it is not limited by molecular size, solubility or lack of long-range order. In the past several years, we have applied magic angle spinning SSNMR based methods to study several protein complexes. In this chapter, we discuss the general solid-state NMR methodologies employed for structural and dynamics analyses of protein complexes with specific examples from our work on thioredoxin reassemblies, HIV-1 capsid protein assemblies and microtubule-associated protein assemblies. We present protocols for sample preparation and characterization, pulse sequences, SSNMR spectra collection and data analysis.

Keywords

SSNMR; magic angle spinning; protein complexes

1. Introduction

Protein-protein interactions are involved in many important biological processes such as signal transduction (¹), cellular transport (²), viral infection (^{3, 4}) and immune response (⁵). These interactions often result in large protein complexes that are insoluble and difficult to crystallize. Due to the insolubility and inherent lack of long-range order in protein assemblies the mature structural techniques that yield atomic-level information, such as solution NMR

¹Distilled water should be used instead of Millipore pure water for preparing M9 medium.

spectroscopy and X-ray crystallography, cannot be applied to studies of such protein complexes. Solid-state NMR spectroscopy has emerged as one of the very few techniques that can yield atomic level structural information for these types of systems. Recently, several studies have been reported on solid-state NMR applications for analysis of protein assemblies, such as bacteriophage viruses (⁶), oligomeric membrane peptides and proteins (^{7–10}), amyloid fibrils (^{11–17}), HIV-1 capsid protein assemblies (¹⁸), microtubule-associated protein assemblies (¹⁹), as well as assemblies of soluble proteins (^{20–22}). The major strength of solid-state NMR spectroscopy is that there is no intrinsic limitation on molecular size or solubility, and long-range order is not required. In large systems where the resonance lines are narrow but spectral congestion presents a challenge, sparse (²³), differential (²⁰), and selective isotopic labeling (^{24, 25}) enables simplification of SSNMR spectra and hence detailed atomic-resolution information can be attained (^{21, 22, 26, 27}). Furthermore, with solid-state NMR spectroscopy residue-specific dynamics can be probed for protein complexes on multiple timescales ranging from picoseconds to many seconds (²⁸), which fosters a deeper understanding of their biological function.

Resonance assignment (or chemical shift assignment) is a prerequisite for extracting site-specific structural and dynamics information in proteins by NMR spectroscopy, including solid-state NMR (²⁹). NMR experiments for resonance assignments generate two types of information. The first is correlations between atoms within the same residue, which allow for amino acid type identification. The second is correlations between atoms belonging to neighboring residues, which allow for establishing sequential connectivities. With the intraresidue and sequential correlations and from the known primary sequence of a protein, site-specific resonance assignments are extracted. In solid-state NMR spectroscopy, either through-space (dipolar) or through-bond (scalar) correlation spectroscopy can be employed for assignments under the MAS conditions (Figure 1 illustrates orientation of the sample rotor with respect to the static magnetic field). Magic angle spinning (³⁰) frequencies of 8–20 kHz are usually employed for multidimensional correlation spectroscopy. In the multidimensional MAS NMR correlation experiments, the typical building blocks for constructing the pulse sequences are i) cross polarization (CP) for ¹H-¹⁵N or ¹H-¹³C polarization transfer, ii) double cross polarization (DCP) (³¹) and its band-selective version (SPECIFIC-CP) (³²) for ¹⁵N-¹³C polarization transfer, iii) PDSO (³³), DARR (³⁴), DREAM (³⁵), RFDR (³⁶), SPC5 (³⁷) and several other sequences for ¹³C-¹³C magnetization transfer through proton-driven spin diffusion or its rotary assisted variant or by direct ¹³C-¹³C dipolar recoupling, iv) TOBSY (³⁸), CTUC-COSY (^{39–41}), and several other sequences for ¹³C-¹³C magnetization transfer through scalar couplings. For reviews of the homonuclear and heteronuclear dipolar recoupling methods see (⁴²), (⁴³) and (⁴⁴), respectively. Figure 2 shows the typical 2D and 3D MAS NMR experiments for NMR assignments based on these building blocks, and the corresponding 2D and 3D spectra for thioredoxin reassembly, CAP-Gly/MT reassembly, and HIV-1 CA assembly are presented in Figure 3.

Differential (²⁰), selective (^{24, 25}) and sparse (²³) isotopic labeling enables simplification of NMR spectra as well as the distinction between intra- and intermolecular correlations. Non-uniform labeling is commonly employed in the structural and dynamics analysis of large protein assemblies by solid-state NMR spectroscopy. Differential labeling with

paramagnetic tags for gaining long-range intermolecular constraints in protein interfaces is another emerging area (^{45–47}). There is quite extensive literature on applications of these various labeling schemes to solid-state protein NMR spectroscopy (^{20, 21, 48, 49}). In this chapter, we discuss one of the possible labeling schemes, namely the differential labeling of two interacting proteins where one molecule is enriched in ¹⁵N, and the second molecule in ¹³C, ¹⁵N. This approach enables detailed structural analysis of the ¹³C, ¹⁵N labeled protein and at the same time extraction of the intermolecular interface information by a suitable dipolar dephasing technique. We employed this labeling protocol in the 1–73(U-¹³C, ¹⁵N)/74–108(U-¹⁵N) thioredoxin reassembly (see section 3.1), and developed a set of 2D MAS NMR experiments, which allow for simultaneous identification of the residues constituting the intermolecular interface and resonance assignment of the binding partners (²⁷). These experiments, REDOR-PAINCP, REDOR-PDSD, REDOR-HETCOR, and HETCOR-REDOR are presented below. Pulse sequences for these experiments are shown in Figure 4 and representative spectra acquired by these experiments are shown in Figure 5.

There are a number of software packages for multidimensional data processing and analysis, such as NMRPipe/NMRDraw (⁵⁰), RNMRTK (⁵¹), NMRView (⁵²), Sparky (⁵³), ccpNMR (⁵⁴), ANSIG (⁵⁵), and, SIFT (⁵⁶). The choice of a particular software package is somewhat judicious as many of these programs offer similar capabilities. In our laboratory, we typically employ NMRPipe for multidimensional NMR data processing and Sparky for spectral analysis. In multidimensional processing, the choice of the processing parameters is determined by the specifics of the experiment, and in some cases, it is beneficial to process the SSNMR spectra in two or more different ways, tailored for either sensitivity- or resolution enhancement. The window functions and other processing functions are applied as necessary. For example, a common processing sequence may include (in one or all dimensions, as needed): 90° or 60° shifted sine bell/sine square apodization followed by a Lorentzian-to-Gaussian transformation (for sensitivity or resolution enhancement, respectively); forward linear predication in the indirect dimension(s), zero filling, phase correction, polynomial or multipoint baseline correction. Depending on a particular experiment, maximum entropy reconstruction (^{57, 58}) and/or non-uniform sampling algorithms (^{56, 59}) may be beneficial.

Numerical simulations of solid-state NMR spectra are an integral part of most of the data analysis protocols. Numerical simulations can be employed for any part of the solid-state NMR investigation, from pulse sequence design to interpretation of anisotropic lineshapes to quantitative calculations of specific spectra. In the past decade, several powerful software packages have been developed for numerical simulations of SSNMR experiments, including ANTIOPE (⁶⁰), GAMMA (⁶¹), BlochLib (⁶²), SIMPSON (⁶³), and SPINEVOLUTION (⁶⁴). In addition to these multi-purpose simulation packages, researchers in the field often use custom-coded programs, for example under Mathematica and Matlab environments. In our work on protein assemblies, we utilize SIMPSON, SPINEVOLUTION, as well as home-written Mathematica- and Fortran- based programs.

Our laboratories have been working on the development of magic angle spinning (MAS) SSNMR spectroscopy for investigation of protein complexes. In this chapter, we present experimental protocols for sample preparation techniques, resonance assignments by MAS

NMR spectroscopy, structure analysis and dynamics studies of protein complexes based on our work on three classes of protein complexes: thioredoxin reassembly, HIV-1 capsid protein assembly and microtubule/CAP-Gly assembly (18, 19, 22, 27, 28). Figure 6 illustrates representative morphologies of HIV-1 CA assemblies, microtubules (MT) and CAP-Gly/MT assemblies before and after magic angle spinning.

2. Materials

2.1. Preparation of thioredoxin reassemblies for solid-state NMR studies

1. Protocols for preparation of thioredoxin reassemblies can be found in reference (68).
2. Luria-Bertani (LB) Media Plates (1 L): Dissolve 10 g of bacto-tryptone, 5 g bacto-yeast, 10 g NaCl and 18 g bacto-agar into 950 mL of tap water. Adjust pH to 7.0 with NaOH. Adjust volume to 1 L with tap water. Sterilize by autoclaving for 20 minutes at 15 lb/sq.in on the liquid cycle. Let it cool to ca. 40 °C to add antibiotics. The final concentration of ampicillin was 0.05 mg/mL. Swirl the solution gently to avoid bubbles and pour plates. Allow plates to cool before inverting them. Store at 4 °C.
3. Luria-Bertani (LB) Liquid Media (1L): Dissolve 10 g bacto-tryptone, 5 g bacto-yeast and 10 g NaCl into 950 mL of tap water. Adjust pH to 7.0 with NaOH. Adjust volume to 1 L with tap water. Sterilize by autoclaving for 20 minutes at 15 lb/sq.in on the liquid cycle. Let it cool to ca. 40 °C to add antibiotics. Add ampicillin to a final concentration of 0.05 mg/mL.
4. M9 Liquid Minimal Medium (1 L): Add 200 mL of 5× M9 salts (1), 2 mL of MgSO₄ (2), 0.1 mL of CaCl₂ (3), 20 mL of 20% glucose (4), 10 mL of 100 mg/mL of NH₄Cl (5) and 1 mL of 50 mg/mL (6) in 767 mL of tap water.
5. M9 salts (5×): Dissolve 64 g of Na₂HPO₄•7H₂O, 15 g of KH₂PO₄ and 2.5 g of NaCl in 500 mL of tap water. Adjust volume to 1 L with tap water. Divide the solution into aliquots of 200 mL. Sterilized by autoclaving for 20 minutes at 15 lb/sq.in on the liquid cycle.
6. MgSO₄ (1 M): Dissolve 1.2037 g of MgSO₄ into 10 mL of tap water and sterilize by filtration.
7. CaCl₂ (1 M): Dissolve 1.1098 into 10 mL of tap water and sterilize by filtration.
8. 20% Glucose: Dissolve 4 g of glucose in 20 mL of tap water. Sterilize by filtration.
9. NH₄Cl (100mg/mL): Dissolve 1 g of NH₄Cl into 10 mL of tap water. Sterilize by filtration.
10. Ampicillin (50 mg/mL): Dissolve 0.5 g into 10 mL of tap water. Sterilize by filtration.
11. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock: 200 mM solution.

12. Size exclusion chromatography buffer: 20 mM sodium phosphate, pH 7.0, 3 mM EDTA. pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
13. Anion exchange chromatography buffer: 20 mM sodium phosphate, pH 7.0, 500 mM KCl, 3 mM EDTA. pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
14. HiLoad Superdex 75 column (*see* Note 2).
15. DEAE-cellulose resin.
16. Citraconylation buffer: 500 mM potassium phosphate, pH is adjusted to 8.5 by titrating with 1 M HCl or 1 M KOH.
17. Citraconic anhydride.
18. 5 M NaOH.
19. Citraconylated thioredoxin purification buffer (size exclusion): 0.5% NH_4HCO_3 , pH is adjusted to 7.9 by titrating with 10%–35% ammonium hydroxide or 1 M HCl.
20. Desalting column: PD-10 disposable column packed with Sephadex G-25 medium resin.
21. Trypsin: Dissolve sequencing grade modified trypsin lyophilized powder in 50 mM acetic acid to 100 $\mu\text{g}/\text{mL}$.
22. 50% Acetic Acid.
23. Sephadex G-25 and Sephadex G-50 resins (*see* Note 3).
24. Denaturing buffer: 10 mM potassium phosphate, pH 7.4, 7.6 M urea, pH is adjusted by titrating with 1 M HCl or 1 M KOH.
25. Refolding buffer: 100 mM potassium phosphate, pH 5.7, pH is adjusted by titrating with 1 M HCl or 1 M KOH.
26. Amicon stirred cell, microcon, membrane with a 3000 Da molecular weight cut off.
27. Precipitation buffer: 35% PEG-4000 in 10 mM NaCH_3COO , 1 mM NaN_3 , pH is adjusted to 3.5 by titrating with 1 M HCl or 1 M NaOH..
28. 10%–35% ammonium hydroxide.
29. 1 M HCl
30. 1 M KOH
31. 1 M NaOH
32. 1 M MgSO_4

²To improve the efficiency of purification, we used chromatography columns purchased from GE healthcare (unless otherwise indicated), which is also the producer of the AKTA FPLC system used in our lab. Columns or resins from other vendors may also work but the procedure details (*e.g.* buffer conditions) will be different and need to be optimized.

³To achieve adequate resolution, the size exclusion column packed with Sephadex G-50 should be long enough (>160 cm for separating two fragments and >140 cm for re-purification).

33. 1 M CaCl₂
34. 20% (w/v) D-glucose
35. 20% (w/v) U-¹³C₆ D-Glucose
36. 4 mm Bruker HRMAS or Varian 3.2 mm thick wall NMR sample rotor
37. *E. coli* BL21 (DE3)

2.2. Preparation of HIV-1 CA assemblies

1. cDNA encoding gag polyprotein, pr55gag: Obtained from the NIH AIDS Research and Reference Reagent Program (²⁰).
2. pET21 vector (EMD chemicals, Inc. San Diego, CA).
3. Basal Vitamins Eagle media.
4. Modified M9 growth medium: Prepared by supplementing the 1 L of standard M9 medium (Section 2.1) with 10.0 mL Basal Vitamins Eagle media (⁶⁶).
5. Growth media for selectively labeling: Prepared by adding a ¹³C, ¹⁵N isotopically labeled amino acid and the other 19 unlabeled amino acids to the cultures at 100 mg/L in M9 medium.
6. IPTG stock: (see Section 2.1).
7. Anion exchange chromatography buffer: 25 mM sodium phosphate, pH 7.0, 1 mM DTT, 0.02% NaN₃, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
8. Cation exchange chromatography buffer A: 25 mM sodium phosphate, pH 5.8, 1 mM DTT, 0.02% NaN₃, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
9. Cation exchange chromatography buffer B: 25 mM sodium phosphate, pH 5.8, 1 M NaCl, 1 mM DTT, 0.02% NaN₃, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
10. Size exclusion chromatography buffer: 25 mM sodium phosphate, pH 6.5, 100 mM NaCl, 1 mM DTT, 0.02% NaN₃, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
11. Anion exchange chromatography column: HiTrap Q HP (GE healthcare)
12. Cation exchange chromatography column: HiTrap SP HP (GE healthcare)
13. Size exclusion chromatography column: HiLoad Superdex 200 (GE healthcare).
14. CA dialysis buffer: 25 mM sodium phosphate pH 5.5, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
15. PEG-20,000 solution: 17.5% in H₂O (e.g. dissolve 1.75 g PEG-20,000 to 8.25 mL water).
16. 10 mM EDTA-Cu(II): Dissolve EDTA-Cu(II) in 90% D₂O/10% H₂O.

17. PEG-20,000/EDTA-Cu(II): Dissolve 1.75 g PEG-20,000 in 8.25 mL 10 mM EDTA-Cu(II) solution.
18. CA tubular morphology incubation buffer: 50 mM Tris buffer, pH 8.0, 1 M NaCl, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
19. 1 M HCl
20. 1 M NaOH
21. Lyophilizer
22. *E. coli* Rosetta 2 (DE3)
23. 4 mm Bruker HRMAS or Varian 3.2 mm thick wall NMR sample rotor

2.3 Transmission electron microscopy of HIV-1 CA protein assemblies

1. TEM staining solution: ammonium molybdate (5% w/v) in water, filtered with 0.2 µm syringe filter.
2. Non-sterile 72-well mini trays with lids.
3. 55mm diameter qualitative circle filter paper.
4. 60mm × 15mm Petri dish.
5. Transmission electron microscope: Zeiss CEM 902, operating at 80 kV.
6. 400 mesh, Formvar/carbon-coated copper grids, stabilized with evaporated carbon films.

2.4 Confocal microscopy of HIV-1 CA protein assemblies

1. One-well chambered cover glasses.
2. Staining solution: 0.5% (w/v) Nile Blue A in water, filtered with 0.2µm syringe filter.
3. Laser scanning microscope: Zeiss LSM 510 NLO (25 mW HeNe laser; 543 nm) equipped with a Zeiss 40× (NA 1.3) oil immersion objective lens

2.5 Cryo-SEM microscopy of HIV-1 CA protein assemblies

1. EM PACT high-pressure freezer (Leica).
2. Gold carrier plates.
3. Copper hats.
4. Gold (for deposition).

2.6 Preparation of CAP-Gly/microtubule complexes

1. Modified M9 growth medium (*see* Section 2.1).
2. IPTG stock (*see* Section 2.1).

3. Buffers for Ni affinity chromatography: 20 mM Tris, pH 7.5, containing 10 mM, 50 mM, 200 mM, or 400 mM imidazole. pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
4. Anion exchange buffer A: 20 mM Tris, pH 7.5, 1 mM DTT. pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
5. Anion exchange buffer B: 20 mM Tris, pH 7.5, 1 M NaCl, 1 mM DTT. pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
6. Microtubule polymerization buffer: 25 mM sodium phosphate, pH 6.0, 25 mM NaCl, 0.4 mM DTT, pH is adjusted by titrating with 1 M HCl or 1 M NaOH.
7. Ni affinity chromatography column: HisTrap (GE healthcare).
8. Anion exchange chromatography column: HiTrap FF-Q (GE healthcare).
9. Lyophilized bovine tubulin powder: Stored at 4 °C. Generally, fresh tubulin solution is used for assays. Excess tubulin solution is quick frozen by liquid nitrogen and stored at -80 °C.
10. Paclitaxel (Taxol): 3 mM paclitaxel dissolved in dimethyl sulfoxide (DMSO). Store at -20 °C.
11. GTP

3. Methods

3.1. Preparation of thioredoxin reassemblies for solid-state NMR studies

For detailed description of overexpression system and the purification protocol of *E. coli* thioredoxin see references (67) and (69). For description of proteolytic cleavage of thioredoxin at Arg-73 by trypsin digestion see reference (68). The salient steps pertaining to the preparation of the solid-state NMR samples of differentially enriched thioredoxin reassembly are outlined below.

1. Prepare differentially enriched thioredoxin reassemblies by overexpressing two batches of thioredoxin in *E. coli* BL21(DE3) separately. Use M9 medium containing $^{15}\text{NH}_4\text{Cl}$ and $U\text{-}^{13}\text{C}_6$ glucose for expression of $U\text{-}^{13}\text{C}, ^{15}\text{N}$ thioredoxin and use M9 medium containing $^{15}\text{NH}_4\text{Cl}$ and natural abundance glucose for ^{15}N thioredoxin (65).
2. Purify each batch of thioredoxin by loading the crude cell extract onto a size exclusion (Superdex 75) column. Elute the protein.
3. Apply the eluant to an anion exchange (DEAE-cellulose) column. Elute the protein.
4. Validate the purity of thioredoxin by using SDS-PAGE and measure its concentration by UV absorbance (extinction coefficient $\epsilon^{280} = 14,100 \text{ M}^{-1} \text{ cm}^{-1}$) (67).
5. Cleave each protein batch at the Arg-73 site by trypsin digestion. First, dialyze thioredoxin against citraconylation buffer and concentrate the protein in an Amicon

stirred cell (molecular weight cut off: 3000 Da) to 0.3 mM. Second, block the lysine side chain amine groups by adding 25 μ L citraconic anhydride at 20 minute intervals (total amount of citraconic anhydride is 50 μ L for every 1 mmol thioredoxin) and allow the reaction to continue for 2 h after the final addition. Add 5 M NaOH to maintain the pH at 8.5. Third, remove excess reagent by running the reaction mixture through a desalting column. Fourth, add trypsin to citraconylated thioredoxin (trypsin to thioredoxin ratio is 1:100 w/w) and allow the enzyme digestion to continue for 6 h at 37 °C. Finally, lyophilize the mixture and incubate in 50% acetic acid for 1 h to remove citraconyl groups on lysine side chains.

6. Separate the two peptide fragments, thioredoxin (1–73) and thioredoxin (74–108), on a size exclusion chromatography (Sephadex G-50) using 50% acetic acid as elution buffer.
7. Purify each fragment separately by size exclusion chromatography (Sephadex G-25) using citraconylated thioredoxin purification elution buffer
8. Validate the purity of each fragment by SDS-PAGE and measure the concentration of each fragment by UV absorbance. Extinction coefficients for the N fragment (1–73) and the C fragment (74–108) are $\epsilon^{280} = 14100 M^{-1} \text{ cm}^{-1}$ and $\epsilon^{215} = 39700 M^{-1} \text{ cm}^{-1}$, respectively.
9. Reconstitute thioredoxin by mixing each ^{13}C , ^{15}N -enriched fragment with its complementary ^{15}N -enriched counterpart. This step results in two reassembled thioredoxin samples: 1–73(U - ^{13}C , ^{15}N)/74–108(U - ^{15}N), and 1–73(U - ^{15}N)/74–108(U - ^{13}C , ^{15}N). Mix equimolar amounts of N and C fragments at low concentration ($\sim 50 \mu\text{M}$) in denaturing buffer, then dialyze against refolding buffer.
10. Concentrate the reconstituted thioredoxin to 70 mg/mL using an Amicon stirred cell and microcon (molecular weight cut off: 3000 Da). Gradually add precipitation buffer (10 μ L every 10 minutes or longer) in 0.5 mL of concentrated thioredoxin solution until no further protein precipitation is observed. Quantify the extent of precipitation by measuring residual absorbance at 280 nm.
11. Centrifuge the hydrated thioredoxin/PEG precipitate at 14,000 g for 15 minutes at 4 °C and transfer the pellet into 4 mm Bruker HRMAS NMR sample rotor or Varian 3.2 mm thick wall NMR sample rotor. Seal the the samples with the upper spacer and the top spinner (*see* Note 4).

3.2. Preparation of HIV-1 CA assemblies

The cDNA encoding gag polyprotein, pr55gag, was obtained from the NIH AIDS Research and Reference Reagent Program (²⁰). The DNA sequence coding for CA (gag residues 133–

⁴To obtain SSNMR spectra with narrow lines, it is critical to preserve conformational homogeneity during the preparation of SSNMR samples. Controlled precipitation is a general protocol that allows for generating conformationally homogeneous SSNMR samples of proteins and protein assemblies that are intrinsically soluble. For controlled precipitation, hanging drop screening is performed first to identify the suitable conditions. In our work, we typically employ polyethylene glycol (PEG) of various molecular weights as the precipitant because in our experience, precipitation conditions for virtually any well-behaved protein or protein complex can be successfully established, leading to high-quality samples. In order to pack more protein sample into the MAS rotor, supernatant should be removed from the protein/PEG pellet generated by controlled precipitation. To preserve conformational homogeneity, the pellet should be kept hydrated.

363) is amplified and subcloned into pET21 vector using NdeI and XhoI sites (⁷⁰). The primers used for PCR amplification are; 5'-GAT ATA CAT ATG CCT ATA GTG CAG AAC ATC CAG GGG-3', and 5'-GTG GTG CTC GAG TCA TCA CAA AAC TCT TGC CTT ATG GCC GGG-3', respectively. Restriction sites are underlined.

1. Express $U\text{-}^{13}\text{C}$, ^{15}N isotopically labeled CA protein in *E. coli* Rosetta 2 (DE3) in modified M9 medium using $^{15}\text{NH}_4\text{Cl}$ and $U\text{-}^{13}\text{C}_6$ glucose as the sole nitrogen and carbon sources. Induce the protein expression with 0.4 mM IPTG at 23 °C for 16 h.
2. Express selectively labeled CA protein in *E. coli* Rosetta 2 (DE3) in M9 medium prepared by adding ^{13}C , ^{15}N isotopically labeled amino acid and the other 19 unlabeled amino acids to the cultures at 100 mg/L when the 0.4 mM IPTG is added for protein expression (see Note 5).
3. Purify the CA protein by anion exchange chromatography using Anion exchange chromatography buffer (see section 2.2). The flow rate is 2 mL/min. The flow through (non-binding part) is collected.
4. Purify the CA protein produced in step 3 by cation exchange chromatography using a gradient formed by cation exchange chromatography buffer A and B (see section 2.2). The flow rate is 2 mL/min. CA containing fraction was eluted at conductivity of ca. 10 ms/cm.
5. Remove aggregates by size exclusion chromatography using the size exclusion buffer (see section 2.2). The flow rate is 2 mL/min.
6. Validate the purity of CA protein by SDS-PAGE and measure the concentration of proteins by UV absorbance (extinction coefficient $\epsilon^{280} = 33,585 \text{ M}^{-1}\text{cm}^{-1}$).
7. Dialyze the CA protein against CA dialysis buffer. For the preparation of CA assemblies containing mixed labels, mix two solutions containing CA protein, each isotopically labeled with a different desired amino acid, in a 1:1 ratio, followed by the assembly step to produce CA assemblies of one of the three morphologies: conical, spherical, or tubular, as described below.
8. Lyophilize the purified CA protein (see Note 6). Prepare CA protein assemblies of conical morphology by adding the PEG-20,000 solution to the lyophilized protein to a final protein concentration of 32 mg/mL. Incubate the mixture for 1 h at 37 °C. Recover the assembled material as the pellet after centrifugation at 18,800 *g* for 5 minutes at room temperature. Pack 15 mg of the precipitate into a 3.2 mm Varian NMR sample rotor and seal the sample with an upper spacer and a top spinner.
9. Prepare the differentially mixed labeled CA samples of conical morphology using PEG-20,000, and if needed for experiments, add EDTA-Cu(II) (see Note 7).

⁵Protein expression is induced only for 2 h 30 min to limit shuffling between labeled and unlabeled amino acids.

⁶To prepare CA assembly of conical morphology, the final concentration should be 32 mg/mL. Lyophilization of CA enables direct preparation of CA solution in PEG-20,000 with an initial concentration of 32 mg/mL.

⁷Introducing 10 mM Cu(II)-EDTA in the precipitant allows the Cu(II)-EDTA complex to diffuse into the CA sample and to enhance proton longitudinal relaxation, thus permitting shorter recycle delays in the NMR experiments under very fast-MAS conditions (MAS frequencies of 40 kHz or greater) (82, 83).

10. Prepare CA assemblies of spherical morphology by mixing a 32 mg/ml CA solution with PEG-20,000 buffer (1:1 volume ratio). Incubate the resulting mixture on ice for 30 min and dilute it 4-fold. Dry the solution containing the spherical assemblies with N₂ gas to remove any excess water. Pack 12 mg of the dried sample into a 3.2 mm Varian NMR sample rotor and seal the sample using an upper spacer and a top spinner (*see* Note 8).
11. Prepare CA assemblies of tubular morphology by incubating the 32 mg/ml CA solution prepared in tubular morphology incubation buffer at 37 °C for 1 h.

3.3. Transmission electron microscopy of HIV-1 CA protein assemblies and CAP-Gly/microtubule protein assemblies

The morphologies of the HIV-1 CA assemblies are analyzed using a Zeiss CEM 902 transmission electron microscope operating at 80 kV. Samples are stained with TEM staining solution, deposited onto 400 mesh, Formval/carbon-coated copper grids, and dried for 40 min. Follow the protocol below to prepare the TEM grids:

1. Transfer 5 µL of CA assemblies slurry and 5 µL of TEM staining solution to two separate wells of a mini tray.
2. Place the copper grid on the CA assemblies slurry first with the shiny side down, incubate for 1 min.
3. Use the edge of a filter paper to remove excess solution.
4. Place the grid on top of the staining solution and incubate for 30 seconds with the same side facing down.
5. Use the edge of a filter paper to remove excess solution.
6. Place the grid on the CA assemblies slurry drop again for 30 seconds with the same side facing down.
7. Use the edge of a filter paper to remove excess solution.
8. Place the grid on the staining solution drop again for 30 seconds with the same side facing down.
9. Use the edge of a filter paper to remove excess solution.
10. Place one piece of filter paper in the Petri dish. Then place the copper grid on the filter paper, which is already in the Petri dish.
11. Place the Petri dish under a lamp to dry the copper grid for 40 minutes.
12. Place the dried copper grid on the TEM sample holder and then acquire the images.

⁸The spherical assemblies are not stable in solution, but are stable and retain their morphology for many weeks when dried under N₂ gas.

3.4. Confocal microscopy of HIV-1 CA protein assemblies

The morphologies of the HIV-1 CA assemblies are analyzed in solution using confocal microscopy. The stain, Nile Blue A is excited under a 543 nm laser line and emits fluorescence in the hydrophobic environment (protein assemblies). For confocal imaging of the CA assemblies, follow the steps below.

1. Place 1 μ L of protein assemblies slurry on a cover glass (*see* Note 9).
2. Add 5 μ L of staining solution to the protein assemblies slurry (*see* Note 10).
3. Acquire the images under 543 nm laser line of 25 mW HeNe laser) scanning microscope using a Zeiss 40 $\times$ (NA 1.3) oil immersion objective lens. Turn on the transmitted light channel when appropriate.

3.5. Cryo-SEM microscopy of HIV-1 CA protein assemblies

The morphologies of the HIV-1 CA assemblies are analyzed using Cryo-SEM microscopy on a cold stage using a high-pressure freezer. The cryo-fixed specimens are cryo-fractured under vacuum to reveal internal structure. For cryo-SEM imaging of the CA assemblies, follow the steps below (*see* Note 11).

1. Place 1 μ L of protein assemblies slurry on the gold carrier plates. Carefully cover the gold carrier plate with copper hat.
2. Transfer the gold plate set to the Leica EM PACT high-pressure freezer and freeze the sample at 2000 bar at $dT/dt > 10000$ $^{\circ}\text{C}/\text{s}$.
3. Transfer the frozen sample to the pre-cooled sample preparation chamber (-125 $^{\circ}\text{C}$) with liquid nitrogen.
4. Fracture the copper cover of the gold cup with the knife in the preparation chamber.
5. Deposit 10 nm of gold on the freshly fractured surface and lower the temperature to -125 $^{\circ}\text{C}$.
6. Transfer the sample to the cryostage for observation and increase the temperature to -90 $^{\circ}\text{C}$ for 5–7 minutes to remove the surface water.
7. Acquire the images at -125 $^{\circ}\text{C}$ and 1.0 kV at a working distance of approximately 4–5 mm.

3.6. Preparation of CAP-Gly/microtubule complexes

CAP-Gly domain of the p150^{Glued} subunit of mammalian dynactin encompassing residues 19–107 was subcloned into the pET28b-His6-SMT3 vector (³⁴) using BamHI and XhoI restriction sites. Successful subcloning was confirmed by DNA sequencing. (SMT-His6)-CAP-Gly containing plasmid was transformed into E. coli BL21(DE3) cells. For subsequent

⁹When observing conical assemblies with confocal microscopy, excess of staining solution is desirable.

¹⁰When confocal microscopy is employed for imaging the tubular CA assemblies, the high salt content quenches some of the fluorescence, and higher receiver gain in fluorescence channel is needed to get good quality images.

¹¹The humidity in the ambient environment is critical for the cryo-SEM experiment. Ice tends to accumulate on the surface and cover the details of the structures when humidity is high.

production of isotopically enriched protein, purification and assembly steps, follow the protocols below.

1. Overexpress $U\text{-}^{13}\text{C},^{15}\text{N}$ (SMT-His₆)-CAP-Gly in M9 medium containing $^{15}\text{NH}_4\text{Cl}$ and $U\text{-}^{13}\text{C}_6$ glucose (see Note 12).
2. Purify the tagged protein by Ni affinity chromatography using the buffers for Ni affinity chromatography (see section 2.6). Remove non-binding and non-specifically-bound protein impurities by wash the Ni-affinity column with buffers containing 10 mM and 50 mM imidazole, respectively. Elute tagged CAP-Gly by a buffer containing 200 mM imidazole.
3. Overexpress His₆-ULP1 protease (71) in LB medium. Purify the enzyme by Ni affinity chromatography (see Note 13) following the same procedure described in step 2. Divide the His₆-ULP1 protease expressed with 1 L LB medium into Eppendorf tubes (0.5 mL/tube) and store at -80°C without assaying enzyme activity.
4. Mix 24 mL (SMT-His₆)-CAP-Gly expressed with 250 mL M9 medium with 1–1.5 mL His₆-ULP1 protease to cleave the SMT-His₆ tag from CAP-Gly (see Note 13). Put the mixture at 4°C overnight.
5. Dilute the mixture (step 4) to 40 mL with 10 mL imidazole buffer and load the diluted mixture to a 5 mL HisTrap (Ni affinity) column. Elute the CAP-Gly^(19–107) with 10 mM imidazole buffer as CAP-Gly^(19–107) does not bind to the column. Elute His₆-SMT3 tag and His₆-ULP-1 with 200 mM imidazole buffer.
6. Purify the CAP-Gly containing fractions by anion exchange chromatography to remove the residual protein and nucleic acid impurities using a gradient formed by anion exchange buffer A and B (see section 2.6). The flow rate is set as 1 mL/min. The gradient is set as 0% to 40% buffer B in 100 mL elution (or 100 minutes time span). The protein is eluted when buffer B is 15%–20% in the gradient.
7. Validate the purity of CAP-Gly^(19–107) by SDS-PAGE.
8. Dialyze $U\text{-}^{13}\text{C},^{15}\text{N}$ CAP-Gly^(19–107) against microtubule polymerization buffer. Concentrate CAP-Gly^(19–107) to 7.3 mg/mL. Measure the concentration of CAP-Gly by UV absorbance ($\epsilon^{280} = 8250 \text{ M}^{-1}\text{cm}^{-1}$).
9. Dissolve bovine tubulin (lyophilized powder) in the microtubule polymerization buffer. Add GTP and paclitaxel to the tubulin solution (final concentration is $30 \mu\text{M}$ for tubulin, 1 mM for GTP and $15 \mu\text{M}$ for paclitaxel). Incubate the mixture at 37°C for 40–45 minutes.

¹²The protocol of Marley, *et al* employs modified M9 medium (30). The timings and the conditions of the individual steps have to be optimized for a specific protein. For CAP-Gly, *E. coli* cells are grown in LB media until O.D. at 600 nm reaches 0.8. Cells are pelleted, washed with M9 medium without a nitrogen or carbon source, and then transferred to the M9 growth medium whose volume is a quarter of that of the LB medium culture. After 1 h of recovery, expression of SMT-His₆-CAP-Gly is induced by addition of IPTG to 0.8 mM . After another 4 hs, cells are harvested for protein purification.

¹³His-ULP1 expression system (in *E.coli*) is a gift from Weill Cornell medical college. ULP1 is a cysteine protease (71). For efficient cleavage by the His₆-ULP1 enzyme, DTT is added into the His₆-ULP1, SMT-His₆-CAP mixture. The final concentration of DTT is 1–5 mM.

10. Mix a 1.5 mL solution of 7.3 mg/mL $U\text{-}^{13}\text{C},^{15}\text{N}$ CAP-Gly^(19–107) with 3.165 ml of 23 μM paclitaxel-stabilized microtubules. Centrifuge the resulting complex at 80,000 g , 4 °C for 40 minutes. Pack 14.2 mg of hydrated gel-like pellets into a 3.2 mm Varian NMR sample rotor and seal the sample using an upper spacer and a top spinner (see Note 14).

3.7. Transmission electron microscopy of CAP-Gly/microtubule protein assemblies

The morphologies of the CAP-Gly/microtubule protein assemblies are analyzed using a Zeiss CEM 902 transmission electron microscope operating at 80 kV. Samples are stained with ammonium molybdate (5% w/v), deposited onto 400 mesh, Formval/carbon-coated copper grids, and dried for 40 min.

1. Polymerize microtubules from bovine tubulin *in vitro* as described in **Section 3.6**.
2. Express and purify natural abundance CAP-Gly according to the procedure described in **Section 3.6**.
3. Gently mix 10 μM CAP-Gly and 10 μM microtubules to prepare CAP-Gly/microtubule assembly (see Note 15).
4. Follow steps 2–12 in **Section 3.3**.

3.8. Solid-state NMR spectroscopy for resonance assignments

The typical SSNMR experimental parameters for 2D and 3D resonance assignment experiments conducted at 14.1 T on a Varian InfinityPlus instrument equipped with 3.2 mm triple tuned T3 probe are detailed below.

1. For the magic angle spinning frequency of 10 kHz, set the radio frequency (rf) field strengths to 95 kHz (^1H), 50 kHz (^{13}C), and 50 kHz (^{15}N) for hard pulses. For $^1\text{H}\text{-}^{13}\text{C}$ CP or $^1\text{H}\text{-}^{15}\text{N}$ CP, contact times are 0.85 and 1.1 ms, respectively; ^1H radio frequency field is 50 kHz, and ^{13}C or ^{15}N radio frequency field is *ca.* 40 kHz in the center of a linear or tangential ramp. Use TPPM decoupling (72); the decoupling field strengths range between 80 and 100 kHz in different experiments. Recycle delays in all experiments are temperature dependent, and for temperatures between 0 and –30 °C are typically set to 2 s.
2. For selective magnetization transfers from ^{15}N to $^{13}\text{C}^\alpha$ (NCA) or to $^{13}\text{C}'$ (NCO), match the ^{15}N and ^{13}C radio frequencies according to $\omega_{\text{N}} \pm \omega_{\text{C}} = n\omega_{\text{r}}$ (32). For example, at 14.1 T and when the MAS frequency is 10 kHz, the typical rf field strengths are: $\omega_{\text{C}} = 25$ kHz (constant amplitude) and $\omega_{\text{N}} = 15$ kHz (at the center of a tangential ramp). The mixing time is 6–7 ms.
3. For the DARR and PDSB sequences employed for the $^{13}\text{C}\text{-}^{13}\text{C}$ correlation spectroscopy either as standalone experiments or as part of 3D NCACX experiment, tune the mixing time to observe cross peaks within the desired range of

¹⁴The CAP-Gly/microtubule ratio is optimized by a co-sedimentation assay (19).

¹⁵Microtubules are fragile protein assemblies and their morphology may be altered during various biochemical manipulations. To prevent shearing of microtubules upon pipetting, 100 μl pipette tips are cut at the sharp end. Prior to and after MAS experiments, microtubule morphologies have to be examined by TEM to ensure that the microtubules remain intact.

distances (*see* Note 16). Typical mixing times for one-bond correlations are 10 ms and 50 ms at 14.1 T and 21.1 T, respectively.

4. Use the DREAM sequence in the 2D/3D NCACB experiment to establish predominantly onebond C^α-C^β correlations following the ¹⁵N-¹³C^α transfer by SPECIFIC-CP. The double-quantum matching condition for the DREAM sequence is $n\omega_r = (\omega_{rf}^2 + \Omega_1^2)^{1/2} + (\omega_{rf}^2 + \Omega_2^2)^{1/2}$ (35). The typical mixing time in the DREAM step is *ca.* 2 ms (*see* Note 17). The one-bond C^α-C^β correlations result in negative cross peaks. Under these conditions, a number of two-bond correlations (*e.g.*, C^α-C^γ correlations for Thr residues) will appear in the spectra, and these are positive-intensity cross peaks.

3.9. Solid-state NMR experiments for probing protein backbone dynamics

The pulse sequences used for backbone dynamics experiments are shown in Figure 7 and described below.

1. ¹⁵N longitudinal relaxation rates (T₁) provide information about motions on the pico- to nanosecond time scales. Insert a $\pi/2$ - τ - $\pi/2$ block into the NCA experiment before the DCP block, by which magnetization is transferred from ¹⁵N to ¹³C^α. Monitor the decay of ¹⁵N magnetization by tracing the peak intensities in a series of 2D NCA spectra (28). Generate relaxation curves from the spectra acquired with a series of delays; for each residue, plot the cross peak intensities as a function of the delay time, and fit the experimental points to a single-exponential function $I = I_0 \exp(-R_1 t)$ to extract the residue-specific relaxation rates R₁ (*see* Note 18).
2. For qualitative detection of submillisecond motions in backbone amide protons, insert a $\tau/2$ - π - $\tau/2$ echo before the ¹H-¹⁵N CP block. Proton magnetization is rapidly dephased during the two $\tau/2$ delays if it is in the rigid environment of the solid protein. Only the amide protons with high mobility on the submillisecond time scale can survive the relatively long delay (*e.g.* 400 μ s), and the backbone nitrogen atoms bonded to these protons detected (*see* Note 18).
3. ¹⁵N chemical shift anisotropy (CSA) is also sensitive to protein dynamics. The CSA is reduced in the presence of motions occurring at frequencies faster than the magnitude of the CSA interaction. Therefore, the ratio of the anisotropy in the presence of dynamics to the static-limit anisotropy as well as the asymmetry parameter of the dynamically averaged CSA tensor are a probe of the amplitude and geometry of the motions. ¹⁵N CSA at 14.1 T is *ca.* 10 kHz, and therefore, it is sensitive to the motions occurring on the time scales faster than 100 μ s. Measure the ¹⁵N CSA tensors site-specifically by introducing a ROCSA CSA recoupling

¹⁶DARR and PDSM mixing times are strongly magnetic field dependent, and polarization transfer at higher fields is slower. The mixing time is determined experimentally for a specific magnetic field strength. For example, for one-bond correlations at 14.1 T, the mixing times are *ca.* 2–10 ms; at 17.6 T- *ca.* 10 ms, at 21.1 T- *ca.* 50 ms.

¹⁷DREAM is a double-quantum homonuclear recoupling sequence, and the magnetization generated by DREAM is of opposite phase of the original polarization. Therefore for NCACB experiment, the N-C^α SPECIFIC-CP should be carefully optimized to avoid two-bond N-C^β magnetization transfer. C^β magnetization generated by non-selective SPECIFIC-CP would cancel signals generated by DREAM.

¹⁸The pulse lengths and power levels are similar to those in experiments for resonance assignments, with additional ¹⁵N T₁ or T₂ filter delays introduced in the corresponding experiments.

block (73) before the ^{15}N chemical shift evolution period in the NCA experiment in a 3D ROCSA-NCA (74) experiment (*see* Note 19). Representative ^{15}N CSA lineshapes are illustrated for reassembled thioredoxin in Figure 8.

4. ^1H - ^{15}N dipolar couplings are also sensitive to motions on the time scales of less than 100 μs . Record residue-specific ^1H - ^{15}N dipolar lineshapes in a 3D DIPSHIFT-NCA experiment by introducing a DIPSHIFT period (75) in the basic NCA sequence. A number of dipolar recoupling sequences can be employed (the original DIPSHIFT (75), TMREV (76), LGCP (77–79)). In our work, we employ an RN-type recoupling block, R18_1^7 (⁴³), for the recoupling of the ^1H - ^{15}N dipolar coupling and at the same time the suppression of the ^1H - ^1H homonuclear dipolar interactions. During the R18_1^7 recoupling period, the ^{15}N chemical shift is refocused by a spin echo (*see* Note 20). Representative ^1H - ^{15}N dipolar lineshapes are illustrated for reassembled thioredoxin in Figure 8.
5. Extract the CSA and dipolar tensor parameters from the 3D ROCSA-NCA and 3D DIPSHIFT-NCA experiments, by numerical simulations of the experimental lineshapes in SIMPSON (⁶³) or SPINEVOLUTION (⁶⁴) to find the best fit to the experimental results.

3.10. Solid-state NMR experiments for structural analysis of protein interfaces

1. The REDOR block (80) is incorporated into a family of NMR pulse sequences to differentiate between ^{15}N nuclei in two distinct environments. REDOR reintroduces the dipolar coupling between ^{15}N and ^{13}C (or between ^{13}C and ^1H) nuclei, which would otherwise be suppressed by magic angle spinning. Therefore, in the differentially enriched 1–73($\text{U-}^{13}\text{C}, ^{15}\text{N}$)/74–108($\text{U-}^{15}\text{N}$) thioredoxin reassembly the ^{15}N or ^1H magnetization in the $\text{U-}^{13}\text{C}, ^{15}\text{N}$ -enriched fragment can be selectively dephased during the REDOR period by the reintroduced ^{15}N - ^{13}C or ^1H - ^{13}C heteronuclear dipolar coupling, respectively, while the ^{15}N or ^1H magnetization in the $\text{U-}^{15}\text{N}$ fragment is retained allowing for subsequent polarization transfer through the interface or within the ^{15}N fragment resulting in either intermolecular or intramolecular isotopically edited correlations, depending on the desired information (*see* notes 21).
2. In the REDOR-PAINCP sequence, the ^{13}C - ^{15}N REDOR period is introduced after the initial ^1H - ^{15}N CP. The residual unwanted ^{13}C transverse magnetization excited by REDOR is removed by the ^{15}N Z-filter, after which only the ^{15}N magnetization of the ^{15}N -enriched fragment is retained. After the ^{15}N t_1 chemical shift evolution period, the ^{15}N magnetization is transferred to ^{13}C using the heteronuclear ^{15}N - ^{13}C PAINCP (11, 76) step followed by the detection of ^{13}C chemical shift evolution in

¹⁹In our experiments, a C2_2^1 POST block (84) is used with $(a, b) = (0.0329, 0.467)$ and one rotor period (100 μs) increment per t_1 point. During ROCSA, a 10 μs ^{13}C π pulse with XY-8 phase cycling scheme (85) is introduced in the middle of every rotor period on the ^{13}C channel, and 110 kHz CW decoupling is employed on the ^1H channel.

²⁰In this study, we used the $\text{R18}_{17} = \{18070180\}_9$ element (⁴³) with 10 kHz MAS spinning frequency.

²¹During the ^{13}C - ^{15}N Rotational Echo Double Resonance (REDOR) dephasing, 100 kHz ^1H TPPM decoupling is employed, and the XY-8 phasing scheme (86) is applied to minimize the resonance offset of the rotor-synchronized π -pulse train. The ^{13}C and ^{15}N radio frequency field strengths are both 50 kHz. Generally the REDOR dephasing time needs to be optimized, and under our experimental conditions the dephasing time longer than 6 ms ensured complete suppression of ^{15}N signals from the $\text{U-}^{15}\text{N}, ^{13}\text{C}$ labeled fragment.

the t_2 period. Since only one of the two fragments ($U\text{-}^{15}\text{N},^{13}\text{C}$) is ^{13}C enriched, the $^{15}\text{N}\text{-}^{13}\text{C}$ cross peaks represent exclusively intermolecular through interface correlations (*see notes 22*).

3. In the PDS-REDOR sequence, a ^{15}N proton-driven spin diffusion (PDS) mixing period is introduced after the initial $^1\text{H}\text{-}^{15}\text{N}$ CP step followed by an ^{15}N t_1 chemical shift evolution period to establish sequential $^{15}\text{N}\text{-}^{15}\text{N}$ correlations. The subsequent $^{13}\text{C}\text{-}^{15}\text{N}$ REDOR dephasing period removes the ^{15}N signals arising from the $U\text{-}^{13}\text{C},^{15}\text{N}$ -enriched fragment, and therefore, in the final spectrum only sequential $^{15}\text{N}\text{-}^{15}\text{N}$ correlations from the $U\text{-}^{15}\text{N}$ labeled fragment can be detected, resulting in considerable spectral simplification due to isotopic editing (*see notes 23*).
4. In the HETCOR-REDOR sequence, the initial part is the FSLG (81)-based $^1\text{H}\text{-}^{15}\text{N}$ HETCOR experiment employing a flat $^1\text{H}\text{-}^{15}\text{N}$ CP with a short contact time to establish one-bond $^1\text{H}\text{-}^{15}\text{N}$ correlations between the amide proton and nitrogen atoms in the entire protein. The ^{15}N magnetization arising from the $U\text{-}^{13}\text{C},^{15}\text{N}$ -enriched fragment is dephased in the subsequent REDOR period, which is introduced after the FSLG-CP part of the sequence. In the final spectrum only $^1\text{H}\text{-}^{15}\text{N}$ correlations from nuclei in the $U\text{-}^{15}\text{N}$ labeled fragment are detected, resulting in considerable spectral simplification due to isotopic editing (*see notes 24*).
5. In the REDOR-HETCOR sequence, the $^{13}\text{C}\text{-}^1\text{H}$ REDOR filter is employed for the dephasing of the ^1H magnetization from the ($U\text{-}^{15}\text{N}\text{-}^{13}\text{C}$) enriched fragment. Under the experimental conditions, ^1H magnetization dephasing is also observed in the part of the ($U\text{-}^{15}\text{N}$) enriched fragment constituting the intermolecular interface. Following the t_1 evolution under FSLG, the ^1H magnetization is transferred to ^{15}N by a flat CP with a short contact time. The ^{15}N signal is detected during the t_2 period. The final spectrum contains the $^1\text{H}\text{-}^{15}\text{N}$ correlations arising solely from the residues of the $U\text{-}^{15}\text{N}$ -enriched fragment, while cross peaks that would be due to the residues constituting the intermolecular interface being either absent or displaying reduced intensity because of their full or partial $^{13}\text{C}/^1\text{H}^{\text{N}}$ REDOR dephasing. A combination of HETCOR/REDOR and REDOR/HETCOR experiments therefore yields information on the intramolecular $^1\text{H}\text{-}^{15}\text{N}$ correlations in the ($U\text{-}^{15}\text{N}$) enriched fragment as well as on the $^1\text{H}\text{-}^{15}\text{N}$ correlations of the residues composing the intermolecular interface (*see notes 25*).

²²During the Proton Assisted Insensitive Nuclei Cross Polarization (PAINCP) transfers (¹¹), the radio frequency field strengths on the ^{13}C and ^{15}N channels are 45 kHz, while the field strength on the ^1H channel is optimized for each experiment and is 57–63 kHz. In the $^1\text{H}\text{-}^{15}\text{N}$ heteronuclear correlation experiments, a flat CP with a short contact time of 170 μs is used.

²³The N–N PDS mixing time utilized in the REDOR–PDS experiments is 4 s. Under these conditions, almost all of the cross-peaks are from the sequential $N_i\text{-}N_{i-1}$ correlations, and the cross peak intensities are 10–30% of the corresponding diagonal signals. The cross peaks from $N_i\text{-}N_{i-2}$ correlations are too weak to be detected.

²⁴The $^1\text{H}\text{-}^1\text{H}$ homonuclear dipolar couplings are suppressed by Frequency-Switched Lee-Goldburg scheme (FSLG) (81), which in the PMLG variant (87) can be implemented by ramping the phase of the proton radio frequency while keeping the proton carrier frequency unchanged.

²⁵ $^1\text{H}\text{-}^{13}\text{C}$ dipolar interaction is 10 times stronger than the $^{15}\text{N}\text{-}^{13}\text{C}$ coupling when the internuclear distances are identical. Therefore, the dephasing effect of $^{13}\text{C}\text{-}^1\text{H}^{\text{N}}$ REDOR is generally stronger than that of $^{13}\text{C}\text{-}^{15}\text{N}$ REDOR. Therefore, the $^{13}\text{C}\text{-}^1\text{H}^{\text{N}}$ REDOR, in addition to dephasing the $^{\text{H}^{\text{N}}}$ signals from the $U\text{-}^{15}\text{N},^{13}\text{C}$ labeled thioredoxin fragment, will give rise to partial dephasing of $^{\text{H}^{\text{N}}}$ signals belonging to the singly ^{15}N labeled fragment and lining the intermolecular interface of reassembled

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Figure 1.

Magic angle spinning: A) sample rotor is spun at an axis which is 54.7° (magic angle) with respect to the static magnetic field; B) a Varian 3.2 mm thick wall rotor is loaded into a Varian T3 probe. The stator (shown in lime green) holds the rotor at the magic angle and allows the bearing/drive air flow to spin the rotor at desired frequency.

Figure 2.

Pulse sequences for resonance assignments of proteins in MAS solid-state NMR: A) 2D ^{13}C - ^{13}C DARR; B) 2D dipolar-based NCA/NCO with SPECIFIC-CP for heteronuclear ^{15}N - ^{13}C polarization transfer; C) 3D dipolar-based NCACX or NCOCX with SPECIFIC-CP and DARR mixing periods for ^{15}N - ^{13}C and ^{13}C - ^{13}C polarization transfers, respectively; D) 3D dipolar-based NCACB with SPECIFIC-CP and DREAM mixing periods for ^{15}N - ^{13}C and ^{13}C - ^{13}C polarization transfers, respectively. Filled and open rectangles represent $\pi/2$ and π pulses, respectively, unless specified otherwise.

Figure 3a

Figure 3b

Figure 3c

Figure 3. Representative solid-state NMR spectra for resonance assignments of protein complexes

A) 2D spectra of the 1–73(U - ^{13}C , ^{15}N)/74–108(U - ^{15}N) thioredoxin reassembly demonstrating the examples of intraresidue and sequential backbone and side chain assignments; A1) ^{13}C - ^{13}C DARR; A2) NCO and A3) NCA. All spectra are recorded at 14.1 T with the MAS frequency of 10 kHz. Reproduced from *Magnetic Resonance in Chemistry* 2007, 45: S73–84 (²²) with permission from John Wiley and Sons.

B) Overlay of 2D DARR spectra of CAP-Gly/MT (black) and CAP-Gly alone (green). The spectra of free CAP-Gly and of CAP-Gly/MT complex are acquired at 21.1 T and MAS frequency of 14 kHz. B2) and B3) are expansions around selected aliphatic regions (C^{α} - C^{β} or C^{α} - C^{γ} correlations) to demonstrate chemical shift perturbations of CAP-Gly upon binding to microtubules. Reproduced from the *Journal of the American Chemical Society*, 2009, 131 (29): 10113–10126 (¹⁹) with permission from the American Chemical Society.

C) Sequential backbone connectivity for the sequence stretch A105–L111 in HIV-1 CA assemblies of conical morphology based on 3D NCOCX, NCACX, and NCACB experiments at 14.1 T and MAS frequency of 10 kHz. The residue names are shown on top of the spectra at their ^{15}N chemical shift plane. Negative cross-peaks resulting from two-bond N - C^{β} correlations in the NCACB spectra are displayed in green. Reproduced from the *Journal of the American Chemical Society*, 2010, 132 (6): 1976–87 (¹⁸) with permission from the American Chemical Society.

Figure 4.

Pulse sequences for interface studies by solid-state NMR. A) ^{15}N - ^{13}C REDOR-PAINCP; B) ^{15}N - ^{15}N PDSD REDOR; C) ^1H - ^{15}N HETCOR-REDOR; D) ^1H - ^{13}C REDOR-HETCOR.

Filled and open rectangles represent π and $\pi/2$ pulses, respectively, unless specified otherwise. XY-8 phase cycle is used in the rotor-synchronous REDOR- π pulse train.

Figure 5.

2D spectra for studies of intermolecular interfaces in 1-73(U- ^{13}C , ^{15}N)/74-108(U- ^{15}N) thioredoxin reassembly: a) REDOR-PAINCP, B) REDOR-HETCOR, C) HETCOR-REDOR, and D) PDSD-REDOR. All spectra are acquired at 14.1 T with the MAS frequency of 10 kHz. Reproduced from the Journal of the American Chemical Society, 2008, 130 (17): 5798-5807 (²⁷) with permission from the American Chemical Society.

Figure 6. Morphology of HIV-1CA assemblies, microtubules and CAP-Gly/MT characterized by confocal and TEM microscopy. A) Confocal images of HIV-1CA assemblies before and after magic angle spinning of the sample; B) TEM images of MT and MT/CAP-Gly assemblies before and after magic angle spinning of the sample.

Figure 7.

Pulse sequences for dynamics studies of proteins and protein assemblies by MAS solid-state NMR. A) 3D ^1H T_2' filtered NCA experiment; B) 3D NCA-based ^{15}N T_1 relaxation experiment; C) 3D DIPSHIFT-NCA experiment with R18_1^7 block employed for dipolar recoupling; D) 3D ROCSA-NCA experiment. Filled and open rectangles represent $\pi/2$ and π pulses, respectively, unless specified otherwise.

Figure 8.

Dynamics information extracted from 3D-ROCSA and 3D-DIPSHIFT experiments in 1–73(*U*-¹³C,¹⁵N)/74–108(*U*-¹⁵N) reassembled thioredoxin. A) Representative ¹⁵N CSA lineshapes; the fit values are: G21, $\delta_\sigma = 34 \pm 2$ ppm, $\eta = 1.0 \pm 0.25$; R73, $\delta_\sigma = 75 \pm 5$ ppm, $\eta = 0.16 \pm 0.11$; V25, $\delta_\sigma = 90 \pm 4$ ppm, $\eta = 0.20 \pm 0.10$; T8, $\delta_\sigma = 97 \pm 4$ ppm, $\eta = 0.20 \pm 0.08$. B) Chemical shift anisotropy δ_σ plotted as a function of the residue number; C) Representative ¹⁵N-¹H dipolar lineshapes; D) Dipolar order parameters $\langle S \rangle$ plotted as a function of the residue number. Reproduced from the Journal of the American Chemical Society, 2009, 131 (38): 13690–13702 (28) with permission from the American Chemical Society.