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Dynamics of Antibody Domains Studied by Solution NMR

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Abstract

Information on local dynamics of antibodies is important to evaluate stability, to rationally design variants, and to clarify conformational disorders at the epitope binding sites. Such information may also be useful for improved understanding of antigen recognition. NMR can be used for characterization of local protein dynamics at the atomic level through relaxation measurements. Due to the complexity of the NMR spectra, an extensive use of this method is limited to small protein molecules, for example, antibody domains and some scFv. Here, we describe a protocol that was used to study the dynamics of an antibody domain in solution using NMR. We describe protein preparation for NMR studies, NMR sample optimization, signal assignments, and dynamics experiments.

Keywords

NMR; relaxation; protein; dynamics; backbone; domain antibody

1. Introduction

Although three-dimensional structures of proteins are mostly obtained by X-ray crystallography, crystal structures are often influenced by crystal packing. This packing reduces local protein dynamics, particularly in solvent-exposed loop regions, or distorts relative domain orientations and protein–protein interactions (1–4). Thus, for an accurate elucidation of structure–activity relationships of proteins, it is useful to characterize protein dynamics and structure by solution NMR (5, 6). Such solution NMR studies also can address whether proteins are intrinsically monomeric or multimeric in solution. In addition, it can provide an evaluation of localized dynamics as a measure of local stability. Such information could be used for rational design of variants with increased stability compared to the original antibody.

In order to study overall protein dynamics and structure by NMR, it is essential to use proteins uniformly labeled with stable isotopes, ^{15}N and/or ^{13}C (7, 8). Such uniform labeling is efficiently achieved by overexpressing the proteins in *Escherichia coli* because proteins can be produced in a minimal medium that contains ^{15}N -labeled NH_4Cl and/or ^{13}C -labeled glucose as the sole nitrogen and/or carbon source(s). Domain antibodies are expressed in *E. coli* and as such they are suitable for such isotope labeling (9–12). NMR studies that can be applied using uniformly labeled proteins are distinct from those for full-size immunoglobulins that contain inherently important oligosaccharides and are therefore typically expressed in eukaryotic systems (13, 14).

Here, we describe media preparation and protein expression protocols adopted from reference (15) with some modification, and describe a basic strategy to perform backbone dynamics studies using solution NMR.

2. Materials

2.1. Protein Preparation

1. *Host cell TG1 E. coli* (Stratagene): Domain antibody is cloned in an expression vector (pHOG) that allows producing the soluble domain antibody proteins in periplasm. For optimal expression, the TG1-competent cells are always freshly prepared for each time of protein induction.
2. 2×TY medium containing 1% glucose and ampicillin.
3. *1 l of ^{15}N minimal medium, pH 7.4*: 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl, 5 g glucose, 1.2 g $^{15}\text{NH}_4\text{Cl}$, 10 mg biotin, 2 mg thiamine, 100 mg ampicillin, 2 ml 1 M MgSO_4 , 1 ml 0.1 M CaCl_2 , and 1 ml mineral salt solution (see Item 5) (15). Fill up to 1 l with sterile water.
4. *1 l of $^{15}\text{N}/^{13}\text{C}$ minimal medium, pH 7.4*: 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 0.5 g NaCl, 2 g D-glucose- $\text{U}-^{13}\text{C}_6$ -labeled glucose, 1.2 g $^{15}\text{NH}_4\text{Cl}$, 10 mg biotin, 2 mg thiamine, 100 mg ampicillin, 2 ml 1 M MgSO_4 , 1 ml 0.1 M CaCl_2 , and 1 ml mineral salt solution (see Item 5) (15). Fill up to 1 l with sterile water.
5. *1 ml of mineral salt solution*: 6 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 6 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.15 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.8 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.7 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 mg $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 mg H_3BO_3 , 0.25 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 5 mg EDTA in water (15).
6. ^{15}N ammonium chloride (Cambridge Isotope Laboratory, Andover, MA) and D-glucose- $\text{U}-^{13}\text{C}_6$ -labeled glucose (Spectra Stable Isotope, Columbia, MD) (see Note 1).
7. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was dissolved in H_2O and filter-sterilized. Stock 0.5 M IPTG is stored at -20°C .
8. *TES resuspension buffer*: 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 20% sucrose, and one tablet protease inhibitor Complete Mini (Roche) per 50 ml solution.
9. *TES/5 osmotic shock buffer*: One volume TES diluted with four volumes of H_2O . Add one tablet protease inhibitor Complete Mini (Roche) per 50 ml water.
10. *Dialysis buffer*: 50 mM Tris-HCl and 0.5 M NaCl pH 8.0 (see Note 2).
11. Amicon concentration filter unit with molecular weight cutoff 5000 Da (Millipore, Billerica, MA).
12. *Immobilized metal affinity chromatography, IMAC*: Hi Trap chelating HP prepacked column, 1 ml (GE Healthcare).
13. *IMAC washing buffer*: 50 mM Tris-HCl and 0.45 M NaCl pH 8.0.
14. *IMAC elution buffer*: 200 mM imidazol in IMAC washing buffer.
15. *Size-exclusion column*: Sephacryl S-200 column.

2.2. NMR Sample Preparation

1. *NMR buffer A*: 2 l of 40 mM phosphate buffer pH 6.0 containing 40 mM NaCl.

^{15}N -labeled sample is prepared using ^{15}N -labeled NH_4Cl in minimal medium. Similarly, $^{15}\text{N}/^{13}\text{C}$ -labeled sample is prepared using both of ^{15}N -labeled NH_4Cl and ^{13}C -labeled glucose in minimal medium. Although we used only protein samples labeled by ^{15}N and $^{15}\text{N}/^{13}\text{C}$, perdeuterated protein samples, that is, uniformly labeled by $^{15}\text{N}/^2\text{H}$ and $^{15}\text{N}/^{13}\text{C}/^2\text{H}$, will be required for NMR studies of antibodies with molecular mass over 30 kDa (20–22). In this case, nonlabile hydrogens in proteins are deuterated using D_2O instead of H_2O in the protein expression medium.

²Optimal buffer condition for purification may be changed depending on each protein.

2. *NMR buffer B*: 2 l of 40 mM phosphate buffer pH 6.5 containing 40 mM NaCl.
3. *NMR buffer C*: 2 l of 40 mM Tris-HCl pH 7.0 containing 64 mM NaCl.
4. *NMR buffer D*: 2 l of 40 mM Tris-HCl pH 7.8 containing 64 mM NaCl.
5. A Slide-A-Lyzer Dialysis Cassette with molecular weight cutoff 10,000 Da and capacity of 0.5–3 ml (Pierce, Rockford, IL), designed for a 2-l beaker.
6. Amicon concentration filter unit with molecular weight cutoff of 10,000 Da (Millipore, Billerica, MA).
7. D₂O with purity >99% (Spectra Stable Isotope, Columbia, MD).
8. A Shigemi NMR tube (Shigemi, Inc., Pittsburgh, PA).
9. A long-tip glass pipette (Wilmad, Buena, NJ).

3. Methods

To characterize protein backbone dynamics, a set of T_1 , T_2 , and $^{15}\text{N}\{-^1\text{H}\}$ NOE experiments for amide nitrogen are often recorded (16, 17). Since there is one backbone amide in each amino acid, except for proline, these experiments provide information on backbone dynamics of an entire protein. Among them, $^{15}\text{N}\{-^1\text{H}\}$ NOE is sensitive to sub-nanosecond motion, and provides qualitative information on internal dynamics in proteins. To obtain backbone dynamics information from $^{15}\text{N}\{-^1\text{H}\}$ NOE, we have three steps in the NMR experiments: (1) optimization of NMR sample conditions, (2) assignment of backbone chemical shifts, and (3) $^{15}\text{N}\{-^1\text{H}\}$ NOE experiments.

Among the three steps, optimization of sample conditions is most critical because of the difference in optimal pH requirements for the domain antibody stability and the NMR studies. Usually, domain antibodies are stable at and above neutral pH. Observation of labile protons (such as amide protons) by NMR is preferred when the exchange with the solvent is slow, that is, in an acid condition. Therefore, pH is chosen to be the minimum value, at which the antibody sample is still stable during the entire NMR experimental procedure.

3.1. Protein Preparation

1. Inoculate single colony of *E. coli* that has been previously transformed with the desired domain antibody in 3 ml 2×TY medium for about 3 h.
2. Check the turbidity, transfer to 1 l 2×TY medium, and continue to incubate at 37°C until OD₆₀₀ ~0.8–0.9. It usually takes about three more hours.
3. Centrifuge to remove 2×YT medium and resuspend the cells into either ^{15}N -labeled medium in two parallel identical procedures as continued below (see Note 1).
4. Add IPTG to make the final concentration 1 mM and incubate at 30°C in shaking incubator, 250 rpm overnight (15–17 h).
5. Harvest the cells by centrifugation at 5000 *g* for 20 min, and resuspend in 10 ml TES buffer for 1 h on ice.
6. Osmotically shock by adding 15 ml of the 5 times diluted TES buffer. Keep on ice for at least 4 h.
7. Centrifuge at 22,000 *g* for 45 min. Collect the supernatant. Dialyze in dialysis buffer overnight at 4°C.
8. Load the dialyzed protein solution into IMAC column.

9. Wash with 10 ml washing buffer supplemented with increasing concentration of imidazol from 0 mM to 40 mM; program the FPLC–IMAC for use of 1 ml HiTrap™ Chelating HP.
10. Elute the antibody with the elution buffer and fractionate the eluate. Program the FPLC flow to 1 ml/min and the collection rate at 0.8 ml/fraction.
11. Load the fractions containing a significant amount of the antibody, that is, the first one or two fractions from IMAC purification (0.8–1.6 ml), into Sephacryl S-200 column. Program the FPLC flow to 1 ml/min and the collection rate at 0.8 ml/fraction and purify the antibody using washing buffer.
12. Concentrate the purified antibody using an Amicon concentration filter by centrifuging at 3000 *g*. Store the antibody as concentrated in washing buffer at –80°C.

3.2. NMR Sample Preparation

1. Take the 400 µl of the purified ¹⁵N-labeled domain antibody at a concentration of 20 µM.
2. A volume of 2 l of the NMR buffer A is stored at 4°C in a 2-l plastic beaker. A quantity of 400 µl of the ¹⁵N-labeled domain antibody is carefully transferred to a Slide-A-Lyzer Dialysis Cassette using a syringe, and dialyzed in the NMR buffer A overnight at 4°C. The volume after dialysis will be ca. 1 ml due to difference in ionic strength between the purified buffer and the NMR buffer.
3. A small amount (~2 ml) of the NMR buffer A in the 2-l beaker is poured into a Amicon filter to equilibrate its membrane for 30 min. After rinsing the membrane, the NMR sample is transferred to the Amicon filter, and concentrated to 280 µl at 4°C. The concentration process takes approximately 1 h when using an Eppendorf Centrifuge 5810R with a swing bucket rotor (A-1-81) at 2500 *g*.
4. The sample concentration is adjusted to 21 µM in 300 µl NMR buffer A. Then, add 16 µl D₂O to make final D₂O content to be 5%.
5. Carefully transfer the NMR sample to an outer Shigemi tube using a long-tip pipette, not to make a bubble in the tube. Slowly insert the inner Shigemi tube, such that no bubbles remain between the inner and outer tubes. The length of the sample portion is set 17 mm assuming use of Bruker NMR instruments. Seal the top of the outer tube by lapping a small portion of parafilm.
6. Steps 1–6 are repeated to prepare each NMR sample in NMR buffers B–D prior to starting an NMR heteronuclear single-quantum coherence (HSQC) experiment (*see Section 3.3.*).

3.3. NMR Sample Optimization by HSQC Experiments

1. These instructions assume the use of a Bruker Avance 600-MHz NMR instrument (14.09 T magnet). They are easily adaptable to other NMR instruments except for names of pulse sequences.
2. Adjust sample temperature to 25°C, and calibrate ¹⁵N pulse width prior to placing the ¹⁵N-labeled protein sample in the NMR magnet.
3. Once NMR sample is in the magnet, turn on deuterium lock, tune and match the probe, optimize shimming, and calibrate ¹H pulse width.

4. Choose the pulse sequence “hsqcfpf3gpplhwg” for a ^1H - ^{15}N HSQC experiment that provides a two-dimensional (2D) heteronuclear chemical shift correlation map between directly-bonded ^1H and ^{15}N in proteins (*see* Note 3). The following parameters are used to run the HSQC experiment. Pulse widths for ^{15}N and ^1H are 45 μs and 10 μs , respectively. Decoupling ^{15}N pulse width is 220 μs . A total of 1024 complex points for an acquisition dimension with a spectral width of 8012 Hz, and 256 complex points for an indirect dimension with a spectral width of 2083 Hz, are recorded with 64 scans (*see* Note 4).
5. Once the NMR spectrum exhibits signals with relatively uniform intensities due to uniform molecular tumbling of a folded protein (Fig. 29.1A), the buffer condition can be used for further NMR experiments. On the other hand, if the NMR spectrum exhibits small peaks and split peaks due to misfolding or instability (Fig. 29.1B), the buffer condition is not desired.
6. Continue Steps 1–5 using NMR buffers B–D to find out the condition such that the pH is minimized but signal intensities are uniformly strong, as shown in Fig. 29.1A. In our case, NMR buffer D, 40 mM Tris buffer at pH 7.8 containing 64 mM NaCl, was the optimal condition.

3.4. NMR Backbone Signal Assignments

1. A $^{15}\text{N}/^{13}\text{C}$ -labeled NMR sample is prepared at 0.5 mM protein concentration in NMR buffer D similarly as described in **Sections 3.1** and **3.2** (*see* Note 5).
2. Once the protein sample is placed in the magnet, turn on the deuterium lock, tune and match the probe, optimize shimming, and calibrate ^1H pulse width.
3. ^{13}C and ^{15}N pulse widths are both calibrated.
4. Two NMR experiments named HNCACB and CBCACONH (18, 19) are run sequentially for backbone signal assignments (*see* Note 6). In the Bruker Avance NMR instrument, the pulse sequences with the names of “hncacbgpwg3d” and “cbcaconhgpwg3d” are chosen for HNCACB and CBCACONH experiments, respectively (*see* Note 7).

³In this HSQC experiment, a pulse sequence that contains water flip-back pulses (23) is applied. This is because the NMR buffer condition required to record antibody signals are in the neutral pH range in which labile amide protons exposed to the solvent rapidly exchange with the solvent protons; otherwise, the sensitivity of the experiment may be lower than that expected.

⁴The number of scans described here assumes use of a 600 MHz NMR instrument with a low-temperature probe that provides ca. three times higher sensitivity than that of an ambient temperature probe. In this condition, a total experimental time to record one HSQC spectrum is 5 h. However, the total experimental time largely depends on protein concentration, magnetic field strength, and performance of the probe. Basically, as molecular size increases, NMR peak height decreases due to slowing down of molecular diffusion. Therefore, a higher protein concentration and/or higher sensitivity NMR equipment (i.e., a high-field magnet or a low-temperature probe) are/is required to study larger proteins. Alternatively, deuterated samples are used to study larger proteins (*see* Note 1).

⁵Since amide nitrogens are sequentially connected through alpha and carbonyl carbons, a $^{15}\text{N}/^{13}\text{C}$ -labeled protein sample is used to assign backbone amide signals. The protein concentration of $^{15}\text{N}/^{13}\text{C}$ -labeled sample can be lower than that (e.g., 0.3 mM) when a high-field magnet (>700 MHz) and/or a low-temperature probe is available. The optimal buffer condition depends on the amino acid composition of the protein sample.

⁶The NMR experiment names denote the observed signal correlations in the spectra. For example, CBCACONH provides correlations of signals from C_β and C_α to NH through C_α , which relates C_β – C_α signals of the ($i-1$)th residue to the NH of the i th residue. HNCACB provides correlations between HN and C_β – C_α in the i th residue and also between NH in the i th residue and C_β – C_α in the ($i-1$)th residue. To assign backbone signals, HNCA may be used instead of HNCACB experiments when sensitivity of HNCACB is low. HNCA provides correlations between HN and C_α in the i th residue and also between NH in the i th and C_α in the ($i-1$)th. Other experiments, such as HNCB, may be applied to increase signal separation, and ^{15}N -edited NOESY may be applied to confirm backbone connectivity.

⁷To assign signals of large domain antibodies, it may need special techniques to reduce the number of observed signals by domain labeling (24, 25) and/or to use specially optimized NMR pulse sequences (26, 27) as well as deuteration (*see* Note 1).

5. In the HNCACB experiment, 56 complex points for a 33-ppm spectral width are recorded in the ^{15}N dimension, and 128 complex points for a 66.7-ppm spectral width are recorded in ^{13}C dimension. Total experiment time when 32 scans are accumulated is ca. 3 days. The same condition, but with 16 scans, is used for the CBCACONH experiment.

3.5. NMR Backbone Dynamics Experiments

1. Use the same $^{15}\text{N}/^{13}\text{C}$ -labeled protein sample that was used to assign signals (*see* Note 8).
2. If the NMR experiment is performed immediately after the assignment, the steps for temperature setting, deuterium lock, shimming, and pulse calibration are not required; otherwise, these steps are performed again.
3. To record the $^{15}\text{N}\{-^1\text{H}\}$ NOE spectra, a pulse sequence “hsqcnoef3gpsi” is loaded (*see* Note 9).
4. The same NMR experimental parameters are used as the $^{15}\text{N}\text{-}^1\text{H}$ HSQC spectra except for the experiment repetition delay, 3 s, at 600 MHz NMR instrument (*see* Note 10).
5. The $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment records two spectra, that is, with and without NOE transfer from amide proton to nitrogen. For each experiment, 1024×256 complex points are recorded with 64 scans. Total experiment time takes approximately 1.5 days.
6. Taking the ratio of peak heights in the two spectra (with and without NOE transfer) gives the NOE value, that is, $\text{NOE} = I_{\text{withNOE}}/I_{\text{withoutNOE}}$ for individual amide peaks (*see* Note 11).

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⁸For the $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment, either ^{15}N -labeled or $^{15}\text{N}/^{13}\text{C}$ -labeled protein can be used. Protein concentration can be lower than 1 mM but should be similar to that used for the assignment experiments. Since the signal-to-noise ratio of the $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment is ca. ten times lower than that of the $^{15}\text{N}\text{-}^1\text{H}$ HSQC experiment, such a high protein concentration is required to obtain data with a sufficient signal-to-noise ratio. Due to this low sensitivity, it is important to achieve good water suppression so the receiver gain is adjusted to maximize the signal-to-noise ratio.

⁹To record the $^{15}\text{N}\{-^1\text{H}\}$ NOE spectra, a pulse sequence that includes water flip-back is preferred (23), which needs a modification of the default pulse sequence.

¹⁰Although 3-s delay for experiment repetition is very long compared with the delays used in HSQC and assignment experiments (1 s), shortening of the delay will result in incomplete recovery of proton magnetization (28–30). Since the delay time for magnetization recovery increases as increase in the magnetic field strength, it is not practical to use NMR instruments at more than 700 MHz for the $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment.

¹¹If T_1 and T_2 experiments for amide ^{15}N are conducted as well as the $^{15}\text{N}\{-^1\text{H}\}$ NOE experiment, model-free analysis (31) is performed (32, 33), which provides more quantitative information on protein backbone dynamics.

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Fig. 29.1. ^{15}N - ^1H HSQC spectra of a domain antibody recorded at (A) pH 7.8 and (B) pH 6.0. In the spectrum B, some signals are split into two peaks, and small sharp peaks are observed, suggesting that there are significant denatured or misfolding species. Peak intensities in the spectrum A are more uniform than those in B.