Journal of Biomolecular NMR, 10 (1997) 289–292
KLUWER/ESCOM

J-Bio NMR 486

High-resolution heteronuclear NMR of human ubiquitin in an aqueous liquid crystalline medium

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Received 5 August 1997
Accepted 11 August 1997

Keywords: Alignment; Bicelle; Dipolar coupling; Lipid; Liquid crystal; ^{15}N relaxation

Summary

A mixture of dihexanoyl phosphatidylcholine and dimyristoyl phosphatidylcholine in water forms disc-shaped particles, often referred to as bicelles [Sanders and Schwonek (1992) *Biochemistry*, **31**, 8898–8905]. These adopt an ordered, liquid crystalline phase, which can be maintained at very low concentrations of the bicelles (down to 3% w/v). At this concentration the spacing between individual bicelles, on average, exceeds 300 Å. The bicelles are shown to have a negligible effect on the rotational diffusion of ubiquitin as judged by the ^{15}N $T_{1\rho}$ values of the backbone amides relative to those in isotropic aqueous solution. The protein exhibits a residual degree of alignment which is proportional to the bicelle concentration, and approximately collinear with ubiquitin's rotational diffusion tensor. The degree of alignment obtained offers unique opportunities for studying the protein's structure and dynamics.

To date, the determination of three-dimensional solution structures of proteins and nucleic acids by NMR relies primarily on the measurement of large numbers of interproton NOEs, supplemented by homo- and/or heteronuclear J coupling information (Wüthrich, 1986; Clore and Gronenborn, 1989; Cavanagh et al., 1995). For non-globular structures, the cumulative error in these very local constraints can make it difficult to determine the relative position of individual domains. Even for globular proteins, the qualitative manner in which interprotein distances are derived from NOEs limits the accuracy at which the time-averaged conformation of biomolecules can be determined locally.

For proteins with a non-zero magnetic susceptibility anisotropy, the small degree of magnetic alignment when placed in a strong magnetic field results in measurable values of the one-bond ^{15}N-^{1}H and ^{13}C-^{1}H dipolar couplings (Kung et al., 1995; Tolman et al., 1995; Tjandra et al., 1996,1997; Tolman and Prestegard, 1996; Tjandra and Bax, 1997). As the internuclear distance for these dipolar interactions is essentially fixed, their value provides direct information on the orientation of the corresponding bond vector relative to the protein's magnetic susceptibility tensor. Therefore, these dipolar couplings constrain the orientation of the corresponding internuclear vectors all relative to the same axis system. These constraints are therefore fundamentally different from the strictly local NOE and J coupling constraints. The addition of less than 100 such dipolar constraints, measured for a small protein complexed with a 16-base-pair DNA fragment, resulted in a nearly twofold reduction of ϕ/ψ pairs outside of the most favored region of the Ramachandran map (Tjandra et al., 1997). It also yielded a different orientation for a loop region for which no long-range NOE constraints were available. Unambiguous validation of the improvement in structural quality for this complex subsequently was obtained by observing the effect of the magnetic field strength on the ^{15}N chemical shifts (Ottiger et al., 1997): whereas only a weak correlation between the field dependence of the ^{15}N shift and the orientation of the corresponding ^{15}N CSA tensor in the molecular frame was observed for the original structure, a much better correlation was observed for the structure calculated with the inclusion of dipolar coupling constraints.

Although the dipolar couplings clearly are extremely valuable for structure calculation, their accurate measurement requires that the protein has a considerably anisotropic magnetic susceptibility tensor. In practice, this

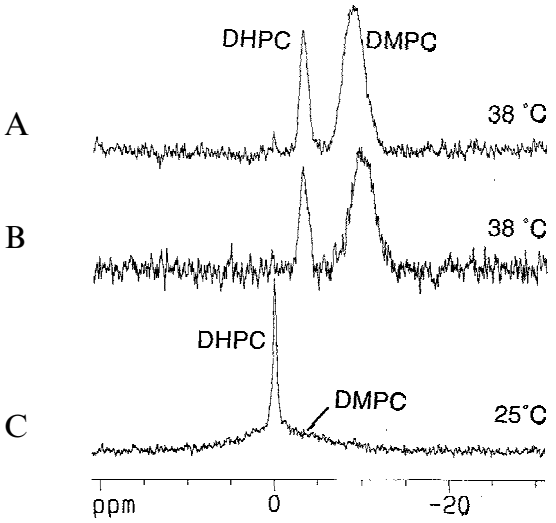

Fig. 1. ^{1}H-coupled ^{31}P NMR spectra of a 2.9:1 molar ratio mixture of DMPC and DHPC in 95% H_2O/5% D_2O: (A) 10% w/v, 38 °C; (B) 3% w/v, 38 °C, (C) 10% w/v, 25 °C. The spectra are referenced relative to the coincident ^{31}P resonances in the unoriented liquid phase, at 10 °C. The downfield resonance corresponds to DHPC and the upfield resonance to DMPC (Sanders and Schwonek, 1992). The ratio of their integrated intensities is 1:2.93.

requires that the molecule is either paramagnetic or contains a large number of coplanar aromatic ring systems, such as found, for example, in double-stranded oligonucleotides. For diamagnetic proteins, the magnetic susceptibility typically is smaller than 10^{-27} cm^3/molecule, resulting in minute dipolar couplings which can be difficult to measure. Various different options for increasing the degree of molecular alignment have been suggested previously, including the use of strong electric fields and polarized light (Tjandra et al., 1997).

Here we demonstrate a technically much simpler method for achieving an adjustable degree of protein alignment: when dissolving a non-spherically symmetric molecule in a solvent containing particles which are oriented relative to the magnetic field, a degree of alignment is transferred to the solute. This constitutes the basis for the study of small molecules dissolved in liquid crystals by NMR (Emsley and Lindon, 1975; Diehl, 1985). Because of the high degree of solute alignment typically obtained in such experiments, a very large number of dipolar couplings result in virtually intractable NMR spectra for solutes containing more than a dozen spins. However, the degree of solute alignment can be reduced by decreasing the concentration of the aligned particles. This reduces the dipolar couplings to an extent that only the largest couplings are observable, thus retaining the resolution, simplicity, and sensitivity of NMR in the isotropic phase.

Experiments were carried out at 600 MHz ^{1}H frequency on a Bruker DMX-600 spectrometer, equipped with a three-axis pulsed-field gradient triple-resonance probehead. The samples used all contain a mixture of dihexanoyl phosphatidylcholine (DHPC) and dimyristoyl phosphatidylcholine (DMPC) in a 1:2.9 molar ratio, dissolved in H_2O. The ubiquitin results presented here were recorded on two samples. The first sample contained 5% w/v DHPC/DMPC and 0.8 mg ubiquitin (0.3 mM) in 300 µl 90% H_2O/10% D_2O, pH 6.9. The second sample was obtained by diluting the first sample by the addition of 125 µl H_2O. In both cases a thin-walled Shigemi microcell (Shigemi Inc., Allison Park, PA) was used. Also, a set of samples containing different concentrations (10%, 7.5%, 5%, 3% w/v) of 1:2.9 DHPC/DMPC were studied by ^{31}P NMR.

Mixtures of DMPC and DHPC in aqueous solvent have been shown to form disc-like assemblies over a substantial range of DHPC/DMPC molar ratios, ranging from 1:2 to 1:3.5 (Sanders and Schwonek, 1992). The DHPC molecules are believed to be located primarily at the edge of the discs, whereas the DMPC molecules form a stable, flat bilayer (Sanders and Schwonek, 1992; Sanders et al., 1994). The DHPC/DMPC molar ratio is believed to determine the size of the discs (Vold and Prosser, 1996). A 1:2.9 molar ratio then corresponds to discs with a thickness of ~40 Å, and a diameter of ~400 Å. These so-called bicelles can form a well-oriented discotic nematic liquid crystalline phase in which the discs are oriented parallel to the magnetic field (Sanders and Schwonek, 1992). The addition of paramagnetic ions can change the sign of the net magnetic susceptibility, causing the bilayers to orient orthogonal to the magnetic field (Prosser et al., 1996). A number of studies on relatively small molecules absorbed in such bicelles have demonstrated that large, structurally informative dipolar couplings can be observed for these solutes (Sanders et al., 1994; Salvatore et al., 1996) and changes in chemical shift when going from the disordered to the liquid crystalline phase

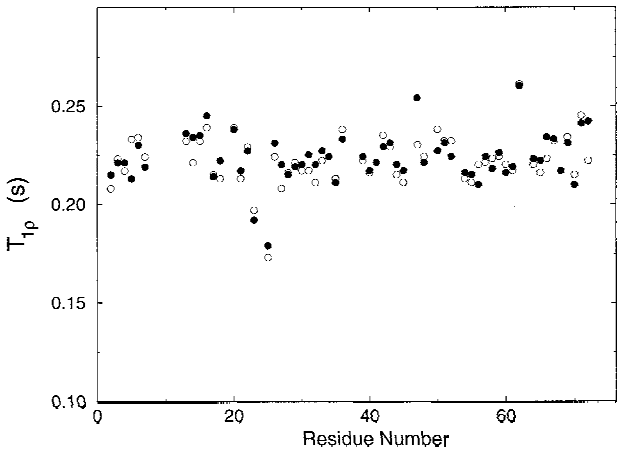

Fig. 2. ^{15}N $T_{1\rho}$ values measured in human ubiquitin at 60.8 MHz ^{15}N frequency, 38 °C, dissolved in 95% H_2O/5% D_2O, pH 6.8. Filled circles: 0.21 mM ^{15}N ubiquitin, 3.5% w/v lipid; open circles: 0.3 mM ubiquitin, no lipid. The strength of the ^{15}N spin-lock field was 2.1 kHz.

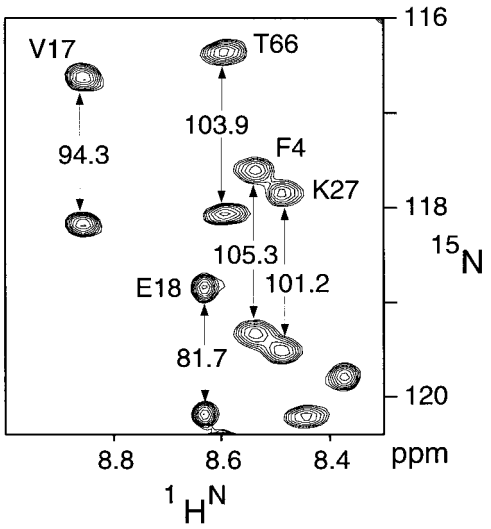

Fig. 3. Small region of the ^{15}N-$^{1}H^{N}$ HSQC spectrum of human ubiquitin (pH 6.8, 38 °C, 0.3 mM, 5% w/v lipid), recorded without ^{1}H decoupling in the t_1 dimension. The $^{1}J_{NH} + {}^{1}D_{NH}$ splittings (Hz) are indicated.

correlate with the orientation of the corresponding CSA tensor relative to the bilayer normal (Metz et al., 1995).

The liquid crystalline phase can be maintained even at very low concentrations of the bicelles, at least down to 3% w/v. Figure 1 shows the ^{31}P NMR spectra recorded at concentrations of 10% and 3% w/v. The ~10 ppm upfield shift of the DMPC ^{31}P resonance in the liquid crystalline phase reflects the orientation of the phosphate group CSA tensor relative to the bicelle normal, and the upfield change in ^{31}P shift indicates that the bilayers are oriented parallel to the magnetic field (Seelig et al., 1985). Clearly, the degree of magnetic ordering of the bicelles is nearly independent of their concentration. The ^{2}H solvent signal shows a well-resolved doublet splitting under these conditions, reflecting incomplete averaging of the large ^{2}H quadrupole coupling in this liquid crystalline medium. The ^{2}H splitting is, to a good approximation, proportionate to the concentration of bicelles, ranging from 16.5 Hz at 10% w/v, to 11.3 Hz at 7.5%, 8.7 Hz at 5% and 5.5 Hz at 3.4%, confirming that the bicelles remain highly ordered.

At the low bicelle concentrations, the average spacing between bicelles exceeds 400 Å, i.e., this spacing is much larger than the size of proteins or nucleic acids typically studied by high-resolution NMR. Even when adding hydration shells of partially ordered water to these bicelles, their spacing remains large relative to the dimension of the solutes. Therefore, rotational diffusion of proteins or nucleic acids dissolved in such a medium is expected to be little affected by the bicelles. Here we report ^{15}N $T_{1\rho}$ relaxation data for human ubiquitin, measured at 3.5% w/v of 2.9:1 DMPC/DHPC in H_2O. In addition, we analyze the degree of magnetic ordering by measurement of the dipolar contribution to the backbone amide ^{15}N-^{1}H multiplet splittings at 5% and 3.5% w/v DMPC/DHPC in H_2O.

Figure 2 shows the ^{15}N $T_{1\rho}$ values as a function of residue at 38 °C. The error in these measurements is relatively large (~5%) due to the low protein concentration (0.3 mM) used in these studies. For reference, the $T_{1\rho}$ values measured in the isotropic phase, on a 0.3 mM sample without DMPC/DHPC, are also shown. Clearly, the ^{15}N $T_{1\rho}$ relaxation times are virtually unaffected by the presence of the bicelles, permitting the recording of high-resolution multi-dimensional NMR spectra.

Figure 3 shows a small region of the ^{1}H-^{15}N HSQC spectrum, recorded at 5% w/v bicelle concentration. The spectrum is recorded without a ^{1}H decoupling pulse in the t_1 dimension, and exhibits one-bond doublet splittings in the F1 dimension, corresponding to the sum of the $^{1}J_{NH}$ scalar and $^{1}D_{NH}$ dipolar coupling constants. Measured values range from 108 Hz for Leu[56] to 76 Hz for Asp[52]. These values differ by up to 18 Hz from the corresponding $^{1}J_{NH}$ values, reported previously, indicating dipolar couplings which are 2 orders of magnitude larger than what is obtained from ubiquitin's magnetic alignment in the isotropic phase at a field of 14 T (Tjandra et al., 1996a). As was observed for the ^{2}H quadrupole splitting of solvent deuterons, the dipolar contribution to the ^{15}N-^{1}H splitting also scales approximately linearly with the bicelle concentration (Fig. 4).

A best fit between the observed ^{15}N-^{1}H dipolar couplings and ubiquitin's X-ray crystal structure (Vijay-Kumar et al., 1987) yields a molecular alignment tensor (Tjandra and Bax, unpublished results), which is nearly collinear with the previously determined rotational diffusion tensor

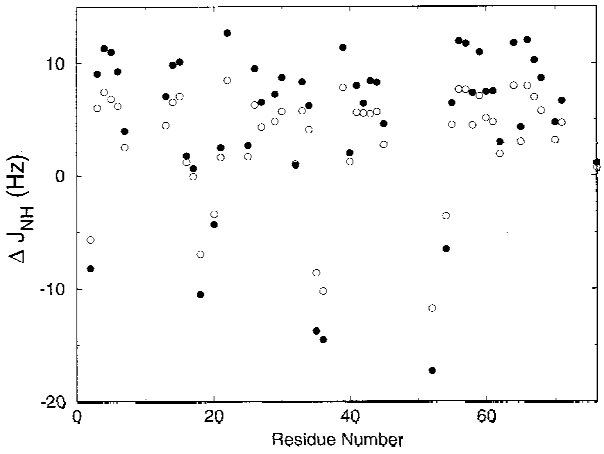

Fig. 4. ^{15}N-$^{1}H^{N}$ dipolar contributions to the $^{1}J_{NH}$ splittings observed in the ^{15}N-$^{1}H^{N}$ HSQC spectrum of human ubiquitin (pH 6.8, 38 °C). Filled circles: 0.3 mM ubiquitin, 5% w/v lipid; open circles: 0.21 mM ubiquitin, 3.5% lipid. The dipolar contributions are obtained by subtracting the $^{1}J_{NH}$ values measured for a 0.9 mM ubiquitin solution in 95% H_2O/5% D_2O. These latter values agree to within 0.3 Hz pairwise rmsd with values reported previously on a different ubiquitin sample (1.4 mM, pH 4.7, 10 mM NaCl, 27 °C) using a time-consuming but more accurate method for measuring these values.

of this protein (Tjandra et al., 1995). This indicates that ubiquitin's molecular alignment is induced primarily by its shape, and not by weak interactions with the bicelles.

Although the degree of protein alignment in the solvent containing oriented bicelles is a function of its shape, most proteins will be sufficiently asymmetric to yield magnetic alignments larger than observed for ubiquitin. In this respect, it is interesting to note that ubiquitin's deviation from isotropic rotational diffusion is unusually small, and the first attempt at measuring it was unsuccessful (Schneider et al., 1992). We have shown that the size of the dipolar couplings can be adjusted by varying the bicelle concentration, such that they fall in a convenient range where their measurement is straightforward. Small degrees of protein alignment, such as used in the present study, appear preferable over the much higher degrees of alignment typically observed for small-molecule solutes in a liquid crystalline phase. The multitude of both short- and long-range dipolar interactions obtained in the case of higher degrees of molecular alignment result in very complex spectra, even in the case of small molecules, and yield unresolvable spectra for the case of macromolecules. The degree of protein alignment obtained at low bicelle concentrations is sufficient to accurately measure one-bond ^{1}H-^{15}N and ^{1}H-^{13}C, and even ^{1}H-^{1}H, ^{13}C-^{13}C and ^{13}C-^{15}N dipolar couplings (Tjandra and Bax, unpublished results). This yields structural information of an accuracy which is unprecedented in macromolecular NMR and promises to increase dramatically the quality of structures obtainable by high-resolution NMR. The method is also expected to be pivotal for studying the solution structure of numerous other types of compounds, including carbohydrates, peptides, and natural products.

Acknowledgements

We thank Marius Clore, Jim Emsley, James Ferretti, Klaus Gawrisch, William Hagins, Jim Hofrichter, Ira Levin, Adrian Parsegian, Donald Rao, Olle Soderman, and Attila Szabo for many stimulating discussions and useful suggestions. This work was supported by the Intramural AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health.

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