19 February 1999

ELSEVIER

Chemical Physics Letters 301 (1999) 138–144

CHEMICAL PHYSICS LETTERS

NMR of biomolecules in low viscosity, liquid CO_2

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Received 29 November 1998

Abstract

We demonstrate that hetero- and homonuclear NMR spectra of cyclosporin A and pancreatic trypsin inhibitor (BPTI) can be recorded at room temperature in liquid CO_2, a medium of very low viscosity, which has been reported to have no adverse effect on molecular structure or activity of several proteins. Rotational diffusion of cyclosporin A in a CO_2/methanol mixture is approximately three-fold faster than in chloroform. Translational diffusion for BPTI, dissolved in liquid CO_2 by co-adding detergent and trifluoroethanol, is measured to be faster than in water, but rotational diffusion is not. Development of improved detergents is anticipated to make feasible the encapsulation of most biological macromolecules in water-containing reverse micelles, which can be suspended in a wide array of apolar, supercritical or near-critical solvents. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The size limit of biological macromolecules amenable to detailed NMR studies is defined primarily by the rapid rate of transverse relaxation of nuclear spin magnetization, which results in large line widths. Although this problem can be alleviated somewhat by the use of extensive deuteration [1], which reduces ^1H–^1H dipolar line broadening, this approach also dilutes the pivotal structural information contained in the ^1H–^1H interactions. Other methods rely on decreasing the transverse relaxation rates through the use of multi-quantum [2,3] and cross-correlation processes [4,5]. Here, we propose the use of an entirely different approach towards decreasing transverse relaxation: the use of a very low viscosity medium. The same approach has been used to study the NMR spectrum of quadrupolar nuclei in small organic compounds [6,7].

In the macromolecular limit, transverse relaxation rates and resonance line widths are directly proportional to the rotational correlation time, and thereby to the viscosity of the medium. Numerous solvents exist which have viscosities more than an order of magnitude lower than the relatively high value of water ($\eta = 1$ cP at 20°C). In particular, many of the solvents with critical points near room temperature, including CO_2, several freons, xenon, and the small hydrocarbons ethane and propane have viscosities below 0.1 cP at room temperature at their vapor pressure. All of these are highly non-polar, and polar molecules such as proteins and nucleic acids are typically very insoluble in these media. Nevertheless, it has been shown that activity of certain enzymes can be retained in an environment of neat CO_2 [8,9],

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PII: S0009-2614(99)00012-3

indicating that protein three-dimensional structure can remain virtually unchanged in such a medium. Also, a range of detergents have been developed which can form emulsions of aqueous microdroplets in such solvents [10–12]. It then is possible to suspend a protein molecule in the aqueous core of such a microdroplet without irreversibly denaturing the protein. The rotational correlation time, τ_c, of such ‘inverted micelles’ is determined by the diameter of this micelle, 2R, and by the (very low) viscosity of the solvent, η:

$$\tau_c = \frac{4}{3}\pi\,\eta\,R^3/kT \qquad (1)$$

where k is Boltzmann’s constant, and T is the temperature. As long as the volume of the droplet divided by the volume of the protein is smaller than the ratio of the water viscosity and that of the near-supercritical medium, τ_c will be reduced relative to that in pure water.

Here, we demonstrate the first NMR measurements on a peptide and a protein in liquid CO_2. The immuno suppressant cyclosporin A is shown to be highly soluble in a 7:1 v/v mixture of CO_2 and methanol without the need for detergents. Pancreatic trypsin inhibitor (BPTI) is solubilized in CO_2 using a fluorinated detergent and trifluoroethanol.

2. Materials and methods

2.1. Preparation of NMR samples

All experiments were carried out using a single-crystal sapphire high-pressure (HP) NMR tube (Saphikon, Milford, NH), with an outer diameter of 5 mm, and a 1 mm wall thickness. A titanium valve, epoxied to the top of this tube, has been described elsewhere [13]. The tube was tested periodically up to pressures of 300 bar; no NMR experiments were carried out at pressures exceeding 100 bar. Owing to the large volume of the liquid CO_2 and the gaseous phase above it, special precautions were taken to shield the pressurized sapphire tube from the operator by a plexiglass cylinder at all times [14].

A clear 21 mM solution of cyclosporin A in 0.4 ml liquid CO_2, methanol-d4 mixture (7:1 v/v) was prepared by condensing CO_2 in the HP–NMR tube, containing a concentrated solution of 10 mg of cyclosporin A in 50 μl of methanol-d4.

Solutions of BPTI were prepared by condensing CO_2 in a HP–NMR tube containing a solution of 3 mg BPTI, 5 μl H_2O, 30 μl trifluoroethanol-d2 (TFE), and 40 mg perfluorinated surfactant, to a final volume of 0.4 ml. Repeated shaking of the tube resulted in an optically clear solution. The surfactant was prepared by bubbling ammonia through a solution of perfluoroheptanoic acid (Aldrich) in chloroform. The white precipitate was filtered and dried under reduced pressure, yielding 70% of the ammonium salt.

2.2. NMR measurements

All NMR experiments were carried out at 20°C and 600 MHz ^1H frequency on a Bruker DMX600 spectrometer, using a triple resonance, three-axis pulsed field gradient probehead. Translational diffusion measurements were carried out as described by Altieri et al. [15]. The gradient strength was varied from 6.8 G/cm to 40.8 G/cm; gradient pulse lengths were 3 ms for the cyclosporin A measurements, and 3 and 4 ms for the BPTI diffusion experiments carried out in CO_2 and D_2O, respectively. The diffusion delay was 25 ms for the cyclosporin A experiments in CO_2 and 100 ms in $CDCl_3$; 100 ms for BPTI in CO_2, and 175 ms for BPTI in H_2O.

^1H–^{13}C shift correlation spectra of cyclosporin A were recorded with the ^1H-detected HSQC experiment, preceded by a BIRD pulse for suppression of t_1 noise. Each spectrum was the result of a 4.5 h measurement.

^{13}C–$\{^1H\}$ heteronuclear NOE values for the $^{13}C^{\alpha}$ resonances in cyclosporin were measured using a one-dimensional ^1H-detected pulse scheme, consisting of a period of ^1H saturation (on/off) followed by a reverse INEPT transfer [16]. Each 1D spectrum resulted from 1280 scans. The delay between scans during which ^1H saturation (using a series of 30-ms spaced 135° pulses) was either on or off, was 3 s.

$^{13}C^{\alpha}$ T1 values were also measured using a set of one-dimensional experiments with ^1H detection [17], and relaxation delays of 15, 120, 235, 340, 445, 550, 655, 760, 865, 920, 1075, and 1180 ms in both CO_2 and $CDCl_3$, and 1280 scans per relaxation delay.

2D NOESY spectra of BPTI in H_2O (pH 3.8) were recorded using the standard NOESY pulse scheme [18]. For suppression of the very broad water signal (exchange with detergent NH_4^+) in the CO_2 medium, short (2 ms) dephasing delays with 180° pulses at their midpoint, were inserted prior to the start of the t_1 evolution and t_2 detection periods. For the spectrum recorded in 95% H_2O, the last 90° pulse was replaced by a WATERGATE pulse combination [19]. Each spectrum shown results from a $200^* \times 512^*$ data matrix, with acquisition times of 28 (t_1) and 72 (t_2) ms. Total measuring time for each NOESY spectrum was 4.4 h. The NOE mixing time was 150 ms.

3. Results

3.1. Cyclosporin A

The ^1H–^{13}C correlation spectrum of cyclosporin A in the 1:7 (v/v) methanol-d4/CO_2 solution is very similar to that in $CDCl_3$ (Fig. 1). Considering that $^{13}C^{\alpha}$ chemical shifts are known to be steep functions of the peptide backbone torsion angles ϕ and ψ, these spectra indicate that the backbone conformation of this cyclic peptide in CO_2 retains a conformation very similar to that in chloroform [20].

The effective viscosity of the methanol-d4/CO_2 solution was measured from the ratio of the translational diffusion rate relative to that measured in $CDCl_3$, which has a viscosity of 0.58 cP at 20°C. The decay of the averaged intensity of all well-resolved backbone *N*-methyl groups as a function of the gradient strength is plotted in Fig. 2A, both for the CO_2 and the $CDCl_3$ medium. The translational diffusion rates obtained in methanol-d4/CO_2 and $CDCl_3$ for cyclosporin A were found to be $14.1 \pm 0.5 \times 10^{-6}$ cm^2/s and $4.6 \pm 0.2 \times 10^{-6}$ cm^2/s, respectively. As translational diffusion is inversely proportionate to the viscosity, η, these results indicate $\eta = 0.24$ cP for the methanol-d4/CO_2 solution.

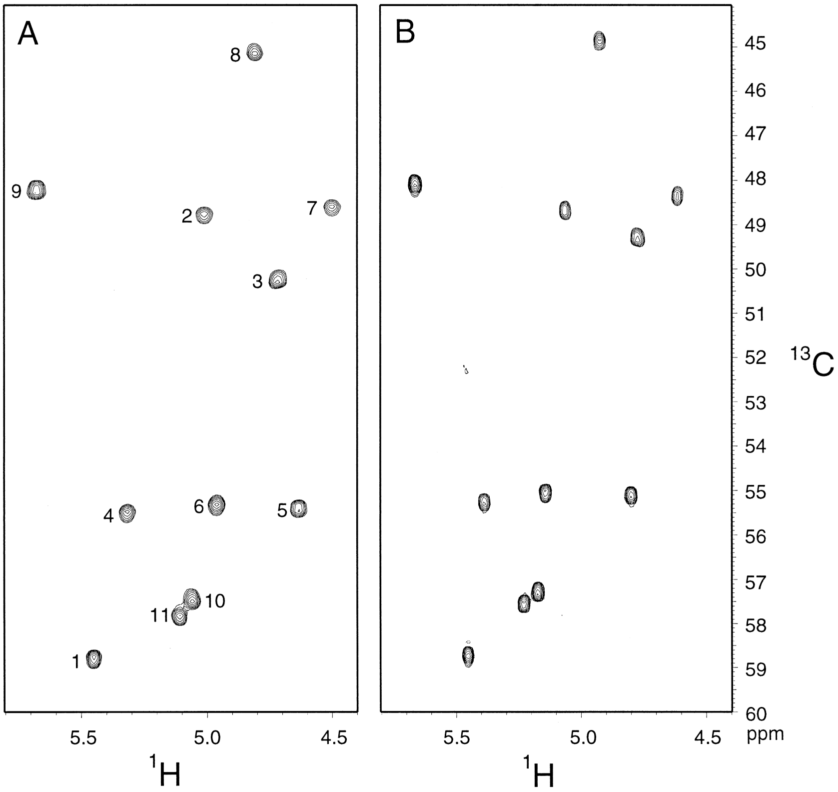

Fig. 1. $^1H\alpha$–$^{13}C\alpha$ region of the ^{13}C–^1H HSQC spectra of cyclosporin A, recorded in (A) $CDCl_3$ and (B) in a 7:1 mixture of CO_2 and CD_3OD. Spectra have been recorded at 600 MHz, 20°C. Numbers in (A) correspond to the residue position in the peptide (see Table 1).

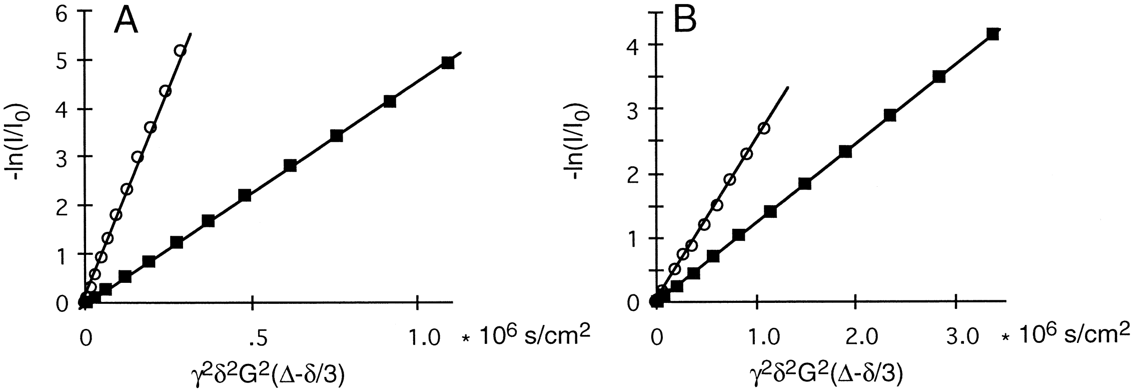

Fig. 2. Translational diffusion data for (A) cyclosporin A and (B) BPTI. Filled squares correspond to $CDCl_3$ (A) and H_2O (B); open circles relate to measurements in the CO_2 containing media. Plotted is the negative natural logarithm of the observed intensity, I, over the intensity observed at zero gradient strength, I_0, in the water-sLED experiment [15], as a function of $\gamma^2\delta^2G^2(\Delta-\delta/3)$, where γ is the ^1H gyromagnetic ratio, δ the gradient pulse duration, G the variable field gradient strength, and Δ the duration between the center of the encoding and decoding gradients.

This viscosity is two-fold higher than the literature value for pure liquid CO_2 at 20°C ($\eta = 0.07$ cP), but 4-fold lower than that of water.

The effect of the solvent on the rotational correlation time of cyclosporin A was evaluated from $^{13}C^{\alpha}$ T_1 and $^{13}C^{\alpha}-\{^1H\}$ NOE measurements (Table 1). Assuming that $^{13}C^{\alpha}$ relaxation is completely dominated by the large, one-bond $^{13}C^{\alpha}$–$^1H^{\alpha}$ dipole–dipole interaction, equations for the longitudinal relaxation rate ($1/T_1$) and NOE are given by:

$$1/T_1 = \frac{h^2\gamma_H^2\gamma_C^2}{40\pi^2 r_{CH}^6}\left[J(\omega_H-\omega_C)+3J(\omega_C)+6J(\omega_H+\omega_C)\right], \qquad (2a)$$

$$NOE = 1 + \frac{\gamma_H[6J(\omega_H+\omega_C)-J(\omega_H-\omega_C)]}{\gamma_C[J(\omega_H-\omega_C)+3J(\omega_C)+6J(\omega_H+\omega_C)]}. \qquad (2b)$$

Assuming rigid body isotropic rotational diffusion, the spectral density function $J(\omega)$ is defined as

$$J(\omega) = \frac{S^2\tau_c}{\left(1+\omega^2\tau_c^2\right)}. \qquad (2c)$$

Other constants: γ_i is the gyromagnetic ratio of spin i; ω_i is the angular Larmor frequency of spin i, h is Planck's constant, and r_{CH} is the effective one-bond internuclear distance (1.117 Å) after correction for the effect of C_α–H_α ultrafast librational motions on the dipolar interaction [21]. Best fitting of the data reported in Table 1 to Eq. (2) yields $\tau_c = 230 \pm 25$ ps in $CDCl_3$ and $\tau_c = 85 \pm 7$ ps in methanol/CO_2. The τ_c value measured in chloroform is nearly two-fold shorter than that previously measured by Dellwo and Wand [22], but this difference is most likely caused by the nearly eight-fold lower peptide concentration used in the present study. The ratio of the τ_c values in $CDCl_3$ and in methanol/CO_2 equals 2.7, in good agreement with the ratio of 3.1 expected

Table 1
Cyclosporin A $^{13}C^{\alpha}$ relaxation parameters in CO_2/methanol and $CDCl_3^a$

Residue	CO_2/CD_3OD		$CDCl_3$	
	T_1 (ms)	NOE	T_1 (ms)	NOE
MeBmt-1	666	2.7	360	2.1
Abu-2	688	2.9	423	2.0
Sar-3	781	3.1	353	2.6
MeLeu-4	745	2.6	–	–
Val-5	716	2.8	495	2.3
MeLeu-6	646	2.8	408	2.3
Ala-7	715	2.5	453	2.3
D-Ala-8	896	2.8	380	2.5
MeLeu-9	708	2.9	334	2.2
MeLeu-10	516	2.8	369	2.1
MeVal-11	722	3.2	355	2.2

[a] Estimated uncertainties in measured parameters are ±50 ms for T_1 in CO_2, ±30 ms for T_1 in $CDCl_3$, and ±0.2 for the NOE in both solvents.

on the basis of the ratio of the translational diffusion constants measured in these media.

3.2. BPTI measurements

Translational diffusion experiments for BPTI were carried out in the same manner as for cyclosporin A. Fig. 2B plots the decay of the BPTI methyl group signal intensity as a function of the diffusion delay for measurements made in 95% H_2O, and in the CO_2/TFE/detergent mixture. The translational diffusion constants derived from fitting these data are 1.21 ± 0.03 and 2.5 ± 0.1 10^{-6} cm^2 s^{-1}, respectively. Thus, these data indicate about 2-fold faster translational diffusion of TFE/detergent-solubilized BPTI in CO_2 than in H_2O. TFE was found to be essential for dissolving the H_2O and BPTI, possibly because the straight perfluorinated alkane chain of the detergent is not suitable for forming a curved monolayer needed for the formation of a spherical inverted micelle.

Fig. 3 shows the NOESY spectrum recorded in CO_2/TFE/water/detergent, using a 150 ms NOE mixing time. On the left side of this spectrum, a narrow strip taken from the NOESY spectrum of BPTI in H_2O and showing the cross peaks for Tyr[23] is shown for comparison. The NOESY spectrum recorded for BPTI in the CO_2/TFE/water/deter-

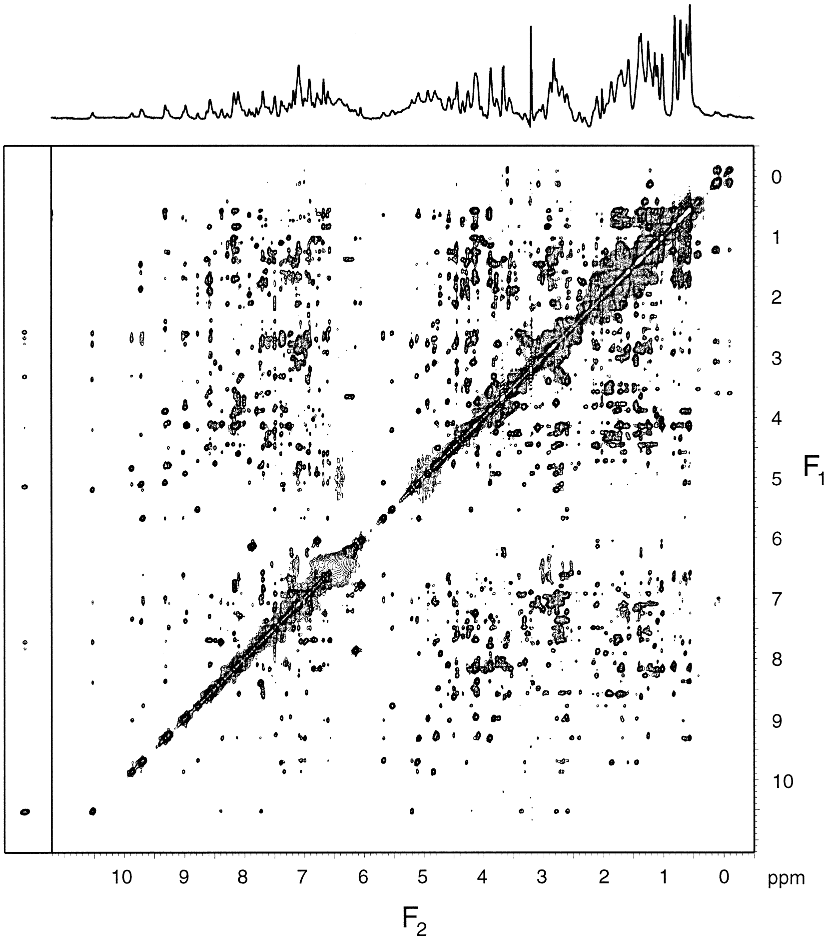

Fig. 3. 600 MHz 2D NOESY spectrum of 3 mg BPTI, detergent-solubilized (40 mg perfluorinated surfactant) in 5 μl H_2O, 30 μl TFE, and CO_2, to a final total volume of 0.4 ml. On the left, a narrow F_1 strip from the NOESY spectrum in H_2O is shown, taken at the chemical shift of the most downfield shifted amide proton (Tyr[23]).

gent mixture is qualitatively similar to that recorded in H_2O at pH 3.8, exhibiting largely the same pattern of cross peaks, but with some changes in chemical shifts of the backbone amide protons relative to those in H_2O solution [23]. Chemical shifts of the aliphatic protons are quite similar in the two media. This indicates that this stable protein retains a very similar conformation in the two media. A comparison of the rotational correlation times can be obtained from the NOE build-up rates for selected cross peaks. These data indicate that NOE build-up in the CO_2/TFE/water/detergent mixture is approximately 1.3 times faster than in H_2O, indicating that the effective rotational diffusion rate is slightly slower in the CO_2 medium compared to water.

The slower rotational diffusion observed for BPTI in CO_2 contrasts with its 2-fold faster translational diffusion. A possible explanation for this puzzling behavior could be that the detergent does not form an ideal, stable inverted micelle, but corresponds to a dynamic equilibrium of a wide distribution of different size micelles which can aggregate and dissociate. The translational diffusion is dominated by the smallest size particles whereas the NOE build-up rate is dominated by the rotational diffusion rate of the largest, slowest tumbling particle. In principle, it should be possible to test this hypothesis by increasing the fraction of CO_2, which would dilute the micelles and thereby reduce their aggregation. However, at higher fractions of CO_2 the protein precipitates, confirming that the CO_2/TFE/water/detergent/protein mixture does not contain ideal, stable inverted micelles.

4. Conclusions

Our measurements indicate that it is possible to record NMR spectra of biological macromolecules in a low-viscosity near-supercritical solvent. Data for cyclosporin A indicate a very substantial increase in rotational diffusion rates. For BPTI, the effect of the larger effective micelle size, relative to the dimensions of BPTI alone, is not offset by the lower viscosity of the solvent. However, for larger proteins the relative contribution of the detergent to the micelle size decreases, and substantial gains in rotational diffusion rate should be obtainable. Equally importantly, it is anticipated that improved detergents, possibly in combination with other inert low viscosity solvents such as freons, small alkanes, or xenon may form more stable, small inverted micelles. The protein is fully surrounded by water in such an inverted micelle and its conformation therefore is expected to remain unperturbed [9,11]. Development of such detergents is a rapidly expanding field of research and it is anticipated that improved detergents will soon be available. We anticipate that this will make it possible to study the solution structure of much larger proteins than is feasible today.

Acknowledgements

SG is the recipient of a predoctoral Fulbright Scholarship.

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