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A Debate on the Use of Presaturation Method in NMR for Structure Determination of Polypeptides in H_2O

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Abstract: Three typical solvent suppression methods employed for measuring the NOE data that are used for structure determination of polypeptides by modeling were discussed and compared with one another. In the experiments with several peptides composed of 10 to 65 amino acids the presaturation method was found to give severely distorted signal intensities of exchangeable protons, thus making the results based on this method much less reliable.

INTRODUCTION

Many important NMR experiments of biological samples are performed in protonated solvents, such as H_2O. This is because exchangeable amino, imino and amide protons may be employed as essential probes for elucidation of the structure and dynamics of biological molecules of interest[1,2]. The detection of relatively weak proton signals from samples in the presence of the intense H_2O signal is usually

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limited by dynamic range problem of detection hardware. There are several traditional methods for overcoming this difficulty. Among them presaturation of the solvent resonance[3] is usually the easiest and the most popular one. Frequency-selective excitation using binomial pulses[4-6], such as the *jump-and-return* (JR) method[6], is widely used for nucleic acid samples. Recently some pulse sequences which combine field gradients and selective excitation pulses have been introduced[7-9].

Many scientists[1,2,10], however, have warned against using the presaturation method in NOE measurements of the exchangeable protons for structure determination, since the magnetization transfer may occur from the labile protons to the solvent protons. This may not be important for structure determination of molecules like protein because most protons in those molecules are rarely exposed directly to the solvent. However, it can be of crucial importance for oligo- and polypeptides which contain less than 30 residues. In this paper whether the presaturation method may be applied for these systems or not has been quantitatively examined by measuring the intensities of the exchangeable proton resonances of four different peptide samples in H_2O. The peptides employed for this purpose are a linear decapeptide hormone called luteinizing hormone releasing hormone (LHRH)[11], an antimicrobial peptide called buforin II[12] from *Bufo Bufo Gargarizans* (21 residues), a section (25 amino acids; 410-434) of Ffh protein from *Escherichia Coli*[13], and a 65-residue-long peptide which is composed of the N-terminal part of $p56^{lck}$(37 amino acids) and the C-terminal part of CD8 (28 amino acids)[14,15].

MATERIALS AND METHODS

Luteinizing hormone releasing hormone (LHRH) of a 98% purity was purchased from Sigma Chemical Co. Inc. (ST. Louis, USA) and was used in our

experiments without further purification. Buforin II and Ffh(410-434) were generously provided by Dr. S. C. Kim and Dr. H. Kim in the Department of Biological Sciences, Korea Advanced Institute of Science and Technology. The N-terminal part of $p56^{lck}$ (37 amino acids) and the C-terminal part of CD8 (28 amino acids) (a 95% purity) were purchased from Chiron Mimotopes (Clayton, Australia) and the $p56^{lck}$-CD8 complex was prepared by letting disulfide bonds formed between them. The formation of the hetero-dimer was confirmed by Ion Spray Mass Spectrometry (Fisons, VG Quattro, data now shown). The samples for NMR measurements were dissolved in 50 mM potassium phosphate buffer at pH 6.7 containing 90% H_2O and 10% D_2O (Aldrich). The concentration of the samples was 5 mM for LHRH and *ca.*1 mM for other polypeptides, respectively.

NMR experiments were performed on a 600 MHz Bruker DMX600 spectrometer at 298K. For the gradient experiments an inverse probehead with an actively shielded Z-gradient coil (Bruker) was used. The data were processed using UXNMR. Water suppression NMR experiments were performed using the original or modified Bruker pulse sequences. 1D NMR spectra were recorded for 8192 data points and the spectral width was 12 ppm. The data were Fourier transformed after multiplication by an exponential window function with the line broadening factor of 1 Hz. Water signal suppression experiments were performed using three different methods :presaturation method, jump-and-return (JR) method[6] and pulsed field gradient (PFG) method. In the presaturation method, the water magnetization was presaturated by irradiating the water peak selectively for one second at 0.0001W transmitter power level before applying the 90° hard pulse. In the JR method, the delay between the two successive 90° pulses was set at 90 μsec so that the exchangeable proton can give as large a signal as possible. For this the length of the second 90° pulse was also adjusted slightly. In the PFG method, the Bruker 3-9-19

pulse sequence was used.[8] The strength of field gradients which were applied before and after the selective 3-9-19 pulse sequence was 10% (5 gauss/cm) of the full gradient power. The gradient pulse length was 1 msec, and the delay for lock recovery was 500 μsec.

RESULTS AND DISCUSSIONS

We used a relatively highly concentrated sample of LHRH (~5 mM) to measure the NMR spectrum in the absence of any water suppression. Fig. 1 shows the ^{1}H NMR spectra of the LHRH obtained with the PFG method (a), the JR method (b), the presaturation method (c), and without any water suppression (d), respectively. In Table 1, normalized relative peak integrals of the 6 backbone NHs (7-9 ppm) of LHRH are listed. Those for other peaks could not be used for the quantitative analysis due to peak overlaps. Integrated area of each peaks was normalized by making use of the peak area of non-exchangeable aromatic ring protons in Tyr-5 (*ca.* 6.75 ppm).

The suppression ratios of the water peak against the non-suppressed water peak are 68 for the case of the presaturation method, 360 for the JR method, and 2200 for the PFG method, respectively(Table 1). In the case of the presaturation method, the solvent signal can be suppressed more than shown in Fig 1-c by increasing the presaturation power. But when this is done, many of the exchangeable proton signals as well as those near the solvent peak disappear. Even at this power level(~0.0001W) the peak at 7.62 ppm is barely observable.

When the presaturation method is used, the integrals of the NH peaks are drastically reduced to about 2% - 55% (98% - 45% reduction) of those for the non-suppressed peaks (Table 1). On the average the reduction ratio of NH peak integrals

in the presaturation case is 26% (74% reduction) of the non-suppressed case, while for the case of PFG and JR method they are found to be 84% and 80%, respectively(Table 1 and 2). The reduction in the signal intensity results from the exchange of the protons between water and the NH protons of the peptide during the presaturation period of 1.7 sec.

Table 1. The Normalized[a] Relative[b] Peak Integrals of Exchangeable Proton Peaks of LHRH.

	solvent suppression methods			
peaks(ppm)	non-suppression	field gradient	JR pulse[c]	presaturation
4.70 (H_2O)	1.0	0.00045	0.0028	0.015
8.42	1.0	0.90	0.80	0.17
8.15	1.0	0.86	0.90	0.27
7.96	1.0	0.84	0.86	0.18
7.87	1.0	0.87	0.83	0.37
7.62	1.0	0.61	0.61	0.02
7.35	1.0	0.93	0.77	0.55

[a]The peak integrals are normalized by the aromatic ring proton of Tyr-5 (6.75 ppm).
[B]relative integrals are the integrals of each method relative to non-suppression method.
[C]For the JR method, the reference peaks are calibrated according to the sinusoidal peak envelope.

Table 2. The statistical analysis of relative peak integrals of the exchangeable proton peaks in Table 1. The average values (Ave), the standard deviations (SD) and the spread of integral value (SIV) of 7 exchangeable NHs in the Table 1 are reported.

	solvent suppression methods		
	field gradient	1-1 pulse	presaturation
Ave	0.84	0.80	0.26
SD	0.11	0.10	0.18
SIV (%)[a]	13	13	69

[a]SIV(%) = 100 x (SD/Ave).

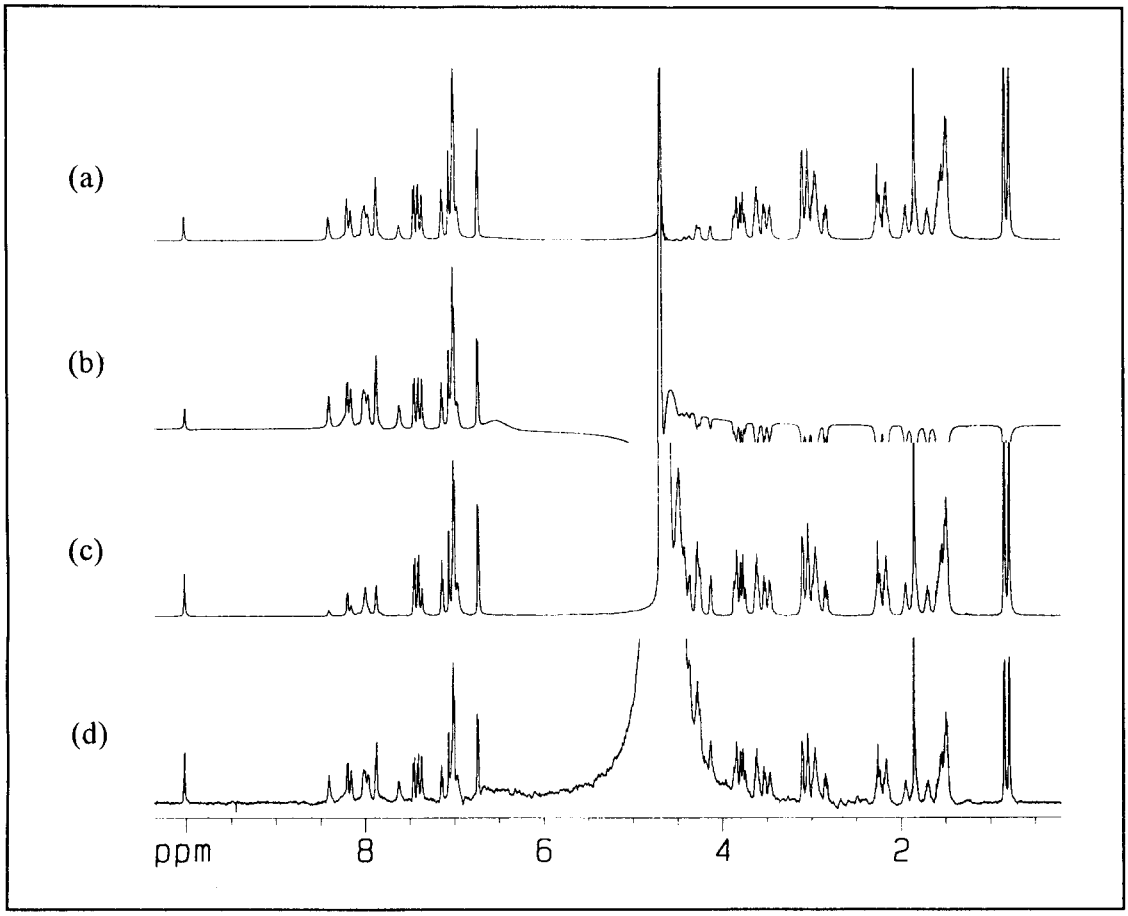

Figure 1. The NMR spectra of LHRH in H_2O/D_2O (90%/10%) acquired by the field gradient method (a), by the JR pulses method (b), by the presaturation method (c) and without any water suppression (d). Each spectrum was scaled by the internal standard peak of the aromatic ring proton of the Tyr-5 (6.75 ppm). The water peak is on the middle of the spectrum (4.7 ppm). The aromatic protons (6.5 - 7.3 ppm) and the exchangeable amide NHs are on the left side of the water peak (> 7.3 ppm).

More serious than intensity reduction itself is the nonuniformity in reduction of amide proton signals caused by the presaturation method. The observed NH integral values were found to spread over 69% of the corresponding average value for the case of the presaturation (Table 2). This is about 5 times higher than observed for

the case of the other two suppression methods. It should be noted that the intrinsic exchange rates of the NH protons vary depending on their own pK values[16], which are affected by the existence of hydrogen bonds and solvent accessibility[17-19]. The neighboring amino acid side chains are also considered to influence the hydrogen exchange rates of amide NHs[20]. Since the amount of the signal reduction varies over various NHs present in the peptide molecule, there seems to be no way of correcting for it unless the proton exchange rate of each NH is measured.

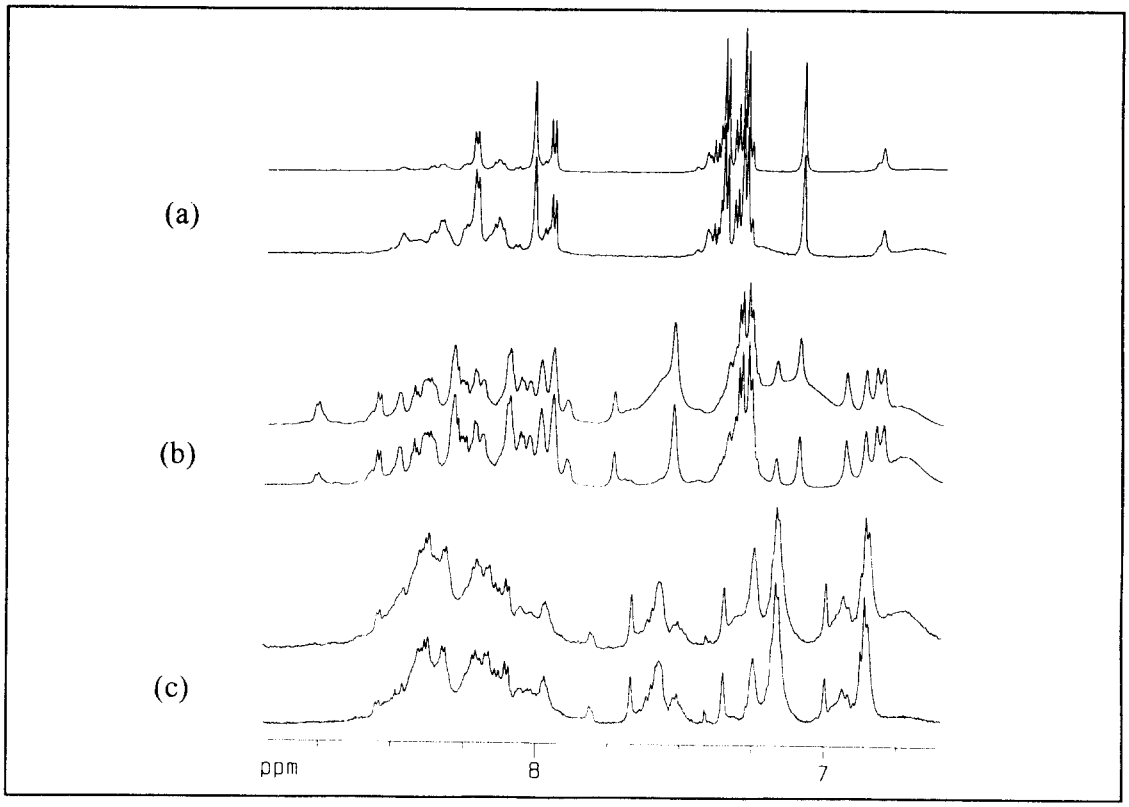

Figure 2. The NMR spectra of Buforin II (a), Ffh(410-434) (b) and $p56^{lck}$-CD8 (c) in H_2O/D_2O (90/10) are shown. The spectra acquired by the presaturation method (below) and the field gradient method (above) are compared in the same scale of their non-exchangeable protons.

We further examined the effect of the presaturation method applied to three other polypeptides which have different sizes, sequences and conformations. Fig. 2 shows how the presaturation affects the intensities of the exchangeable proton resonances for the three samples, Buforin II[12], Ffh (410-434)[13] and $p56^{lck}$-CD8[14,15]. It shows that the sizes of the exchangeable proton peaks are reduced quite a lot and the reduced amounts are different from resonance to resonance as was noted for LHRH.

The presaturation method has widely been used to suppress the water peak in 2D NOESY experiments for oligo- and polypeptide samples and the cross peak intensities of thereby obtained NOESY spectra have been used to draw structural information. Since the NOEs obtained in this way could be reduced by as much as 74% on the average (Table 2), the overestimated internuclear distances would be deduced from such data. To make the matter worse the reduction ratio varies from crosspeak to crosspeak, which would result in severely distorted molecular structure when modeled on the basis of NOE data. Thus we assert that the NOE data of the exchangeable protons obtained by the presaturation method should in no way be used for quantitative purposes.

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