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Suppression of the Solvent Resonance in 2D NMR Spectra of Proteins in H_2O Solution

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The abundance of publications on techniques for recording one-dimensional high-resolution ^1H NMR spectra of biological macromolecules in aqueous solution (*1–5*) clearly indicates that the importance of such experiments for biological applications of ^1H NMR has long been recognized. Specifically for ^1H NMR studies of peptides and proteins, fundamental considerations show that the amide protons play a pivotal role for both the resonance assignments and the determination of the spatial structure (*6*). While in favorable cases certain amide protons in proteins exchange sufficiently slowly with the solvent to be studied in D_2O solution (*4*), investigations extending over the entire polypeptide chain will in all cases have to depend on data recorded in H_2O. Therefore, once the important advantages of two-dimensional (2D) NMR over conventional NMR experiments for studies of proteins were demonstrated with experiments in D_2O (*7–10*), it was of utmost importance to devise experimental schemes by which 2D NMR data sets could be recorded for protein solutions in H_2O. The present note reports on our experience with the use of different solvent suppression schemes for such experiments.

Figure 1 illustrates four different experiments, A to D, that we have used to suppress the H_2O solvent line in ^1H 2D NOE spectroscopy (NOESY) of proteins. In all these experiments a mixed solvent of 90% H_2O/10% D_2O was used and the system was locked on the deuterium line of D_2O. Figure 1A recounts the first scheme proposed for water suppression (*11*), where selective, time-shared irradiation of the solvent resonance ("homodecoupler mode" of the Bruker HX 360 spectrometer) was employed, with a decoupler power of 0 db. In experiment B (Fig. 1) a selective, continuous irradiation of the water resonance is applied at all times except during the observation period t_2, with the decoupler power set between 20 and 30 db. The scheme of Fig. 1C corresponds to experiment B, except that in addition time-shared irradiation of the H_2O line during the observation time t_2 is applied. The fourth experiment (Fig. 1D), finally, foresees continuous irradiation of the water with a low power setting of 20 to 30 db at all times except during t_1 and t_2.

Useful data were recorded with each of the four switching cycles of Fig. 1 (*12–16*), but the practical applications also revealed some disadvantages of schemes A to C. On our instruments the time-shared irradiation during t_2 (Figs. 1A and C) introduced strong, spurious bands of spikes along ω_1 and the cross peaks were bleached

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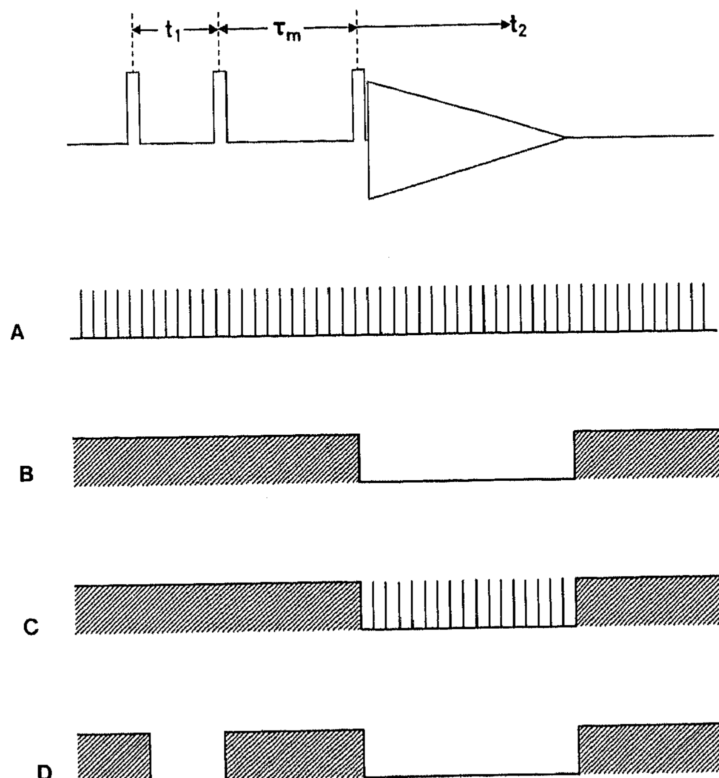

FIG. 1. Experimental schemes used for solvent suppression in NOESY spectra of proteins in H_2O solution. The top trace indicates the sequence of three nonselective 90° pulses of the NOESY experiment (*9*), where t_1, τ_m, and t_2 are, respectively, the evolution period, the mixing time, and the observation period. The traces A to D illustrate four different switching cycles for the selective irradiation of the water resonance. ⊥⊥⊥ indicates that time-shared irradiation ("homodecoupler mode") was applied, /// indicates continuous irradiation, and └──┘ indicates that the decoupler was turned off.

out in a sizable portion of the spectrum near the water line (Figs. 1 and 2 of Ref. (*11*), Fig. 7 of Ref. (*12*)). Since the available decoupler power is more efficiently used than in the time shared irradiation (Fig. 1A), continuous irradiation with scheme B of Fig. 1 resulted in satisfactory suppression of the solvent line with much lower power settings, provided the solvent line was intrinsically narrow and the field homogeneity was carefully adjusted. As an illustration of a successful application of this experiment Fig. 2 shows a NOESY spectrum of the inhibitor E from *Dendroaspis polylepis polylepis*, a protein of molecular weight 7000 (*15*). It can be seen that nearly complete suppression of the water line was achieved with conditions where only a very small spectral region near the water line was bleached out (*11*) and minimal spurious noise was introduced. Similar data were obtained in a variety of experiments with different proteins at temperatures between 25 and 80°C (*13–16*). When working at temperatures between 5 and 25°C, where the water resonance is considerably broader, higher decoupler power was needed for satisfactory solvent suppression. These experiments then revealed a serious shortcoming of the scheme of Fig. 1B, since the water irradiation caused Bloch–Siegert shifts (*20*) along ω_1 of protein resonances located near the water line. This is illustrated in Figs. 3 and 4. In Fig. 3B a NOESY spectrum of the basic pancreatic trypsin inhibitor (BPTI), a protein of mo-

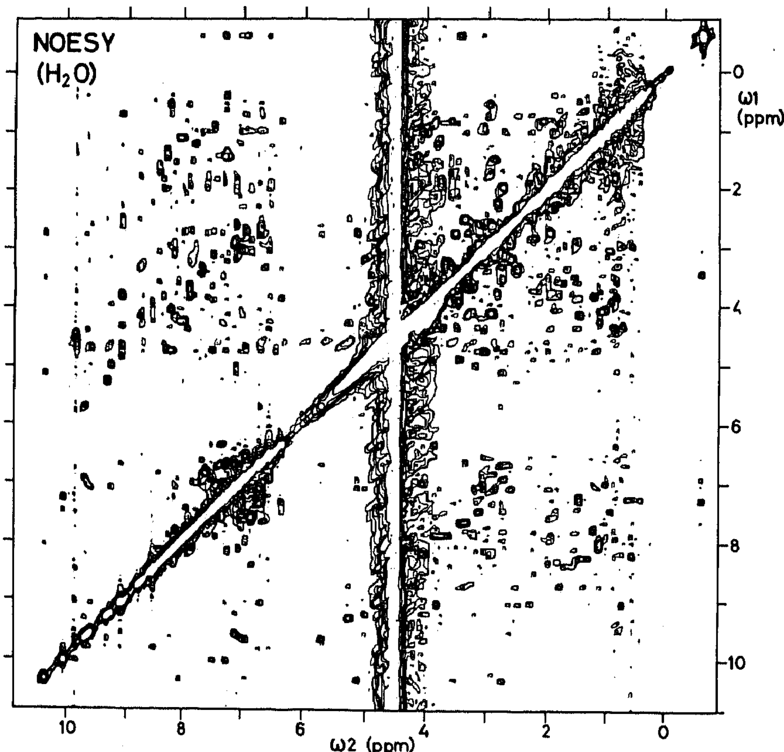

FIG. 2. Contour plot of a 500-MHz ^{1}H NOESY spectrum of a 0.007 *M* solution of *Dendroaspis polylepis polylepis* trypsin inhibitor E in H_2O, pH 3.2, $T = 45$°C. The digital resolution is 5.3 Hz/point. The spectrum was recorded in approximately 22 hrs and a mixing time of 200 msec was used. The strong vertical noise band at 4.5 ppm is at the chemical shift of the water, which was suppressed by selective irradiation as described by the scheme of Fig. 1B.

lecular weight 6500, was recorded with high decoupler power in order to demonstrate the effect of the water irradiation on the diagonal resonances. It is readily apparent that in the region from approximately 3.5 to 4.5 ppm the diagonal peaks are displaced upfield along ω_1, whereby the displacements are largest for the peaks nearest to the residual solvent line. Between approximately 4.5 and 5.5 ppm corresponding downfield displacements of the diagonal peaks along ω_1 can be seen. Identical upfield and downfield shifts in the ω_1 direction prevail for NOESY cross peaks in corresponding ω_1 positions. The appearance of Bloch–Siegert shifts for cross peaks is illustrated in Fig. 4B with the spectrum of another small protein, cardiotoxin $V^{II}2$ from *Naja mossambica mossambica*, which has a molecular weight of 6500. Comparison of Figs. 4A and B shows that the observed shifts depend on the irradiation power and Fig. 3A demonstrates that these shifts do not arise when the irradiation scheme of Fig. 1D is used. Since in NOESY experiments with proteins one uses relatively short t_1 values, typically in the range from 0 to 50 msec *(13–16)*, nearly complete suppression of the water line can be achieved also with this modification of the experiment, where the decoupler is turned off during both t_1 and t_2 (Fig. 3A).

The above considerations for NOESY apply also for correlated spectroscopy (COSY), which uses the pulse sequence *(17–19)*

$$[90°\text{–}t_1\text{–}90°\text{–}t_2]_n.$$

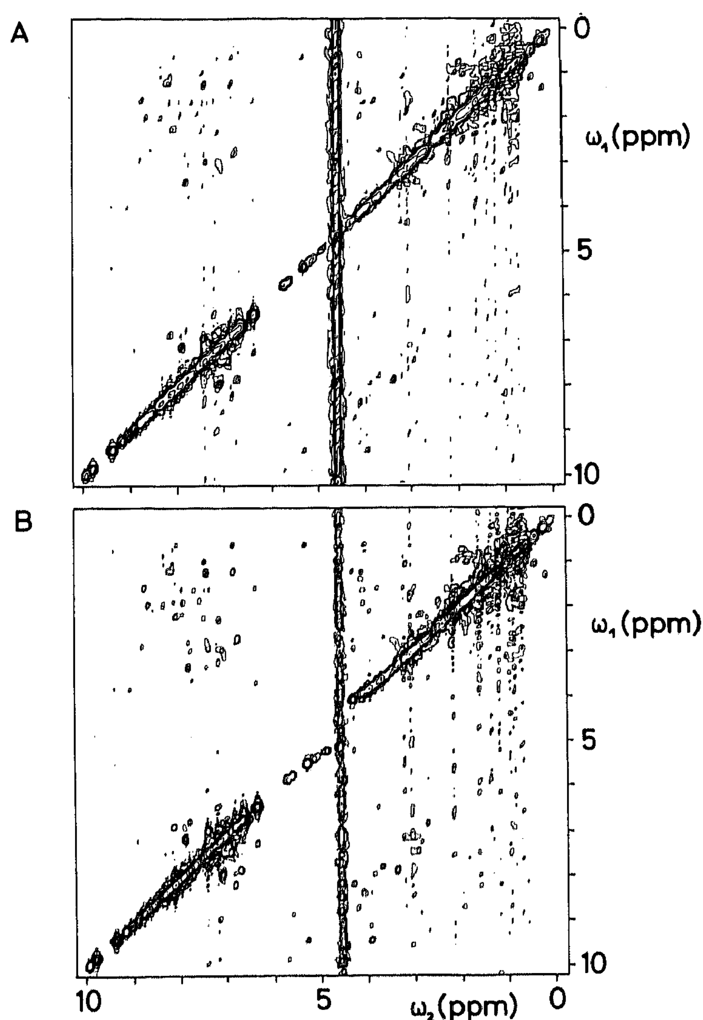

FIG. 3. 360-MHz ^{1}H NOESY spectra of a 0.02 *M* solution of basic pancreatic trypsin inhibitor (BPTI) in a mixed solvent of 90% H_2O/10 D_2O, pH 3.5, $T = 45°C$. The spectra were recorded with a mixing time of 100 msec in approximately 8 hr. A. Suppression of the water resonance with scheme D (Fig. 1) using a decoupler setting of 5 db. B. Water suppression with scheme B (Fig. 1), decoupler setting 5 db. This figure is shown to illustrate the influence of the irradiation during t_1 on the diagonal peaks. Due to the short accumulation time relatively few cross peaks are seen at the level of the contours plotted, and near the chemical shift of H_2O the cross peaks are bleached out by the water irradiation.

When the four different schemes of Fig. 1 were used with COSY (*11–16*), the results were very similar to those described above for NOESY.

In conclusion, our experience so far indicates that for recording NOESY and COSY spectra of proteins in H_2O solutions, the experimental scheme of Fig. 1D, where a selective, continuous irradiation of the water resonance is applied at all times except during the evolution period t_1 and the observation period t_2, is a good approach. To obtain satisfactory results in practice, it is important that the field homogeneity is optimally adjusted, so that effective solvent suppression can be achieved with low power levels and the C^{α} proton lines are bleached out (*11*) essentially only in positions under the water resonance. More elaborate water suppression schemes may be advisable for studies in systems with more rapidly exchanging labile protons, e.g., protein

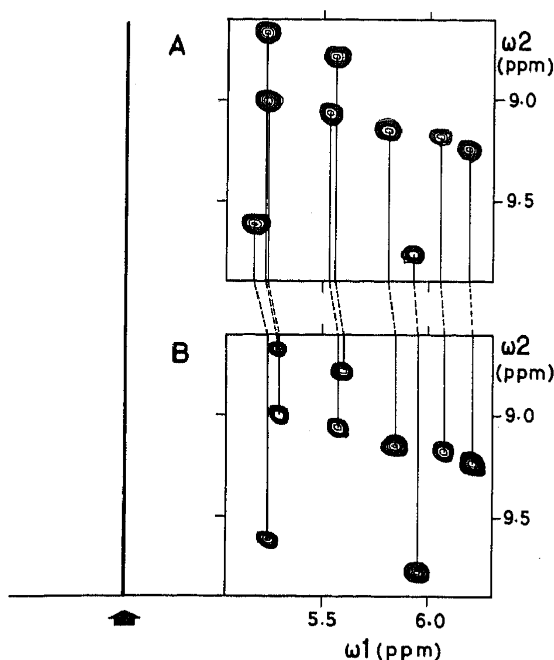

FIG. 4. Contour plots of the spectral region (ω_1 = 5.0 to 6.3 ppm; ω_2 = 8.6 to 9.9 ppm of two 500-MHz ^1H NOESY spectra of cardiotoxin $V^{II}2$ from *Naja mossambica mossambica*. The spectra were recorded with 1×10^{-2} *M* solutions of the protein in a mixed solvent of 90% H_2O and 10% ^2H_2O at pH 3.6 and T = 45°C, and with a mixing time of 100 msec. The water resonance was suppressed by continuous irradiation at all times except during the detection period (Fig. 1B). The decoupler power was 30 and 8 db for spectra A and B, respectively. The heavy vertical line and the fat arrow on the left indicate the frequency of the water irradiation on the ω_1 axis. The peaks in the two spectra correspond to $C^{\alpha}H$–NH NOE connectivities. The solid vertical lines are drawn through the peak centers, corresponding peaks in the two spectra are connected by broken lines.

solutions at high pH or nucleic acid solutions. A recently proposed NOESY experiment where the third 90° pulse (Fig. 1) was substituted by a Redfield-type pulse (*3*), which would selectively excite the signals on the low-field side of the water resonance (*21*), or a variation thereof, might be suitable in such situations.

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