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A Two-Dimensional Nuclear Overhauser Enhancement Experiment Using Semiselective Soft Pulses, and Its Applications to Proteins

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Suitably shaped soft pulses (*1–3*) have frequently been used in high-resolution NMR for the purposes of semiselective excitation. They can be employed to obtain one-dimensional analogs of two-dimensional sequences (*1, 4*) and to record 2D spectra with reduced spectral width in the ω_1 dimension (*5*). This approach also facilitates the recording of 3D NMR spectra in a reasonable measurement time (*6, 7*). Soft pulses, however, have rarely been used as mixing pulses. Only one example has been reported (*8*) in which preparation and mixing in a COSY experiment were achieved with soft pulses in order to restrict coherence transfer to connected transitions only, resulting in the appearance of cross peaks with a pattern of the E.COSY (*9*) or *z*-COSY (*10*) type.

In this communication we describe a soft NOESY experiment consisting purely of semiselective soft pulses to selectively observe NOEs between amide protons in a protein. Because the solvent signal is only minimally excited, soft NOESY spectra can be recorded with short mixing times (<80 ms) on samples containing up to 90% H_2O even in the presence of a broad water signal. Because of a phenomenon known as radiation damping (*11, 12*), this is difficult to achieve with other techniques that do not employ presaturation of the water resonance. This is of practical importance in both protein and nucleic acid NMR, as it is often the case that presaturation leads to the disappearance of NH resonances via saturation transfer due to rapid exchange of NH protons with solvent. Further, the soft NOESY is especially suitable for the detection of cross peaks close to the diagonal since parallel transitions appear with much lower amplitudes. Finally, under suitable conditions, the soft NOESY experiment enables one to obtain higher resolution spectra in a shorter period of time for a fixed number of transients per increment than would be possible using a classical nonselective experiment (*5*).

For various technical reasons, one may hesitate to furnish a pulse sequence completely with semiselective pulses. These include baseline distortions, huge phase gradients, and eventually distortions of the lineshape of the excited signals. More severe problems occur when soft pulses are applied to a large area of the spectrum as the excitation profile differs from that of a 90° pulse at positions far away from the carrier

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frequency. This gives rise to complicated mixing processes and may lead to cross-peak amplitudes and structures within the cross-peak multiplets that are difficult to interpret. Despite the associated difficulties, semiselective pulses offer a number of distinct advantages which render them particularly useful in certain situations, as discussed below.

The sequence described here is the same as that of the classical NOESY sequence (*13*), except that the three hard pulses are replaced by three Gaussian-shaped soft pulses (Scheme [1]) calibrated to a flip angle of 90° on resonance (denoted as $\pi/2^s$):

$$\pi/2^s\text{—}t_1\text{—}\pi/2^s\text{—}\tau_m\text{—}\pi/2^s\text{—}t_2. \qquad [1]$$

All three pulses may be applied at the same frequency yielding a subspectrum containing the diagonal. Alternatively, the last pulse may be applied at a different frequency from the first two pulses, thereby enabling a subspectrum of another spectral region to be obtained [e.g., the $NH(\omega_2)$-$C^\beta H(\omega_1)$ region]. The pulse length we chose to excite the NH region was 1.6 ms. In order to minimize effects on the lineshape due to broad lines and to avoid inordinately large phase shifts, the ratio of the pulse length to dwell time should be no greater than ~6.

An alternative sequence is

$$\pi/2^s\text{—}\pi\text{—}\Delta\text{—}t_1\text{—}\Delta\text{—}\pi\text{—}\pi/2^s\text{—}\tau_m\text{—}\pi/2^s\text{—}t_2. \qquad [2]$$

Here the undesired effects on the baseline of the first two pulses are compensated with π pulses following and preceding the evolution time (*7*). This approach is more straightforward insofar as only minimal phase correction is necessary in ω_1. Scheme [1], however, has superior characteristics with respect to the nonexcitation of the water resonance, thereby permitting NOESY spectra with shorter mixing times to be recorded. In addition, the minimum phase cycle for the first sequence (eight scans) is four times shorter than that for the second.

The essential difference with respect to the usual hard pulse NOESY experiment is that the magnetizations are created with an amplitude corresponding to the flip angle of the pulses at the frequency of the signals. These flip angles vary as the effect of offset increases. In the following discussion, we will assume for simplicity that the flip angle β is constant over the whole multiplet of one spin, but different for different multiplets. The flip-angle dependence may then be expressed by $\beta(\omega)$, indicating that β is a function of ω. Accordingly, a pulse about the y axis converts I_z to I^-:

$$I_{kz} \xrightarrow{\beta(\omega_k)I_{ky}} I_k^- \sin \beta(\omega_k).$$

Similarly, single-quantum coherences are converted into populations by a pulse whose flip angle depends on ω:

$$I_k^- \xrightarrow{\beta(\omega_k)I_{ky}} -I_{kz} \sin \beta(\omega_k).$$

The fact that the flip angle of the mixing pulses is not 90° over the whole spectral range leads to two effects: (a) the integrated amplitude of a whole cross peak may be attenuated, and (b) the individual components of cross peaks or diagonal peaks may be reduced in amplitude or disappear.

The first effect can be discussed using a two-spin system without couplings as an example. In this case we are solely concerned with the exchange of z magnetizations that are excited with different amplitudes. At the beginning of the mixing process of a NOESY experiment with semiselective pulses, the z magnetizations of spins k and l are excited by the factors $-I_{kz}\sin^2\beta(\omega_k)$ and $-I_{lz}\sin^2\beta(\omega_l)$, respectively. Thus, the initial conditions, expressed in vector form, are

$$\mathbf{I}_z\,(t = 0) = \begin{bmatrix} -I_{kz}\sin^2\beta(\omega_k) \\ -I_{lz}\sin^2\beta(\omega_l) \end{bmatrix}.$$

The $\sin^2\beta$ term arises from the fact that there are two pulses preceding the mixing time. The changes in populations occurring during the mixing time are then governed by the equation (*14*)

$$\frac{d\mathbf{I}_z}{dt} = -\mathbf{R}(\mathbf{I}_z - \mathbf{I}_z^0).$$

The matrix **R** has the form

$$\mathbf{R} = \begin{pmatrix} \rho_k & s_{kl} \\ s_{lk} & \rho_l \end{pmatrix}.$$

A further factor of $\sin\beta(\omega_l)$ for the amplitude of the last pulse leads, in the initial rate approximation, to an integrated amplitude of a cross peak between two spins of

$$a_{kl} \approx \sigma_{kl}\sin^2\beta(\omega_k)\sin\beta(\omega_l)\tau_m,$$

where l is the spin detected. The intensity of the diagonal peak a_{ll} is given by

$$a_{ll} \approx (1 - \rho_l\tau_m)\sin^3\beta(\omega_l).$$

Because of the $\sin^3\beta$ term, it is clear that even a spurious excitation of the water resonance leads only to a small water signal on the diagonal. Additionally, the spectra will not be symmetrical (*15*).

In the presence of couplings, multiplet effects will occur, depending on the extent of excitation of the individual spins. There are two limiting cases: namely, the coupled spin is either fully excited or not excited at all. Our example corresponds to the latter, where the NH region is excited and the region containing the coupled $C^\alpha H$ protons does not experience significant excitation. For vanishingly small mixing times, the parallel transitions in the diagonal multiplets of the NH protons are absent because the process is of the type $I^-I^\alpha \rightarrow I^-I^\beta$ and will therefore only take place if there is a pulse on the passive spin. With a nonvanishing mixing time, this process may become "relaxation allowed" due to transitions between the two pairs of eigenstates ($I^\alpha I^\alpha/I^\beta I^\alpha$ and $I^\alpha I^\beta/I^\beta I^\beta$) (*16*).

The soft NOESY experiment with 1.6 ms Gaussian pulses is illustrated on a sample of 6 mM α_1-purothionin (*17*) in 90% H_2O/10% D_2O. The pure-phase absorption spectrum, recorded using the time proportional incrementation (TPPI) method (*18*) and exciting exclusively the NH region, was obtained in ~7 h with 212 increments (Fig. 1A). For comparison, the corresponding region of a conventional NOESY spectrum

obtained in ~24 h (512 increments and TPPI) with preirradiation of the water resonance is shown in Fig. 1B. Both spectra were recorded with a mixing time of 200 ms. The limiting resolution [defined as $0.6/t_{1\,max}$ or $0.6/t_{2\,max}$ for the ω_1 and ω_2 dimensions, respectively (*5, 19*)] is comparable for the two spectra in ω_2 (3.5 and 4.1 Hz for the soft and hard NOESY, respectively). In ω_1, however, it is significantly better for the soft NOESY spectrum (8.4 Hz versus 16.5 Hz for the hard NOESY).

A comparison of the two spectra shown in Fig. 1 indicates that their quality is comparable, although in this particular example the resolution in ω_1 is clearly superior for soft NOESY. Thus, the variation of the pulse flip angles that occurs in soft NOESY does not prevent cross peaks from being observed within a relatively large spectral region. Additionally, a small but welcome improvement arises as a result of the narrower diagonal in soft NOESY, thereby permitting better observation of cross peaks close to the diagonal. Unfortunately, some of the weak NOE cross peaks at the edges of the soft NOESY spectrum are not visible at the contour level plotted, although they can be observed in the appropriate cross sections.

In conclusion, the soft NOESY experiment described here provides a useful addition to the arsenal of techniques available to the protein and nucleic acid NMR spectroscopist. In particular, it permits one to record spectra of samples in H_2O with shorter mixing times than would otherwise be possible, and to obtain, in favorable cases, spectra with a narrower diagonal.

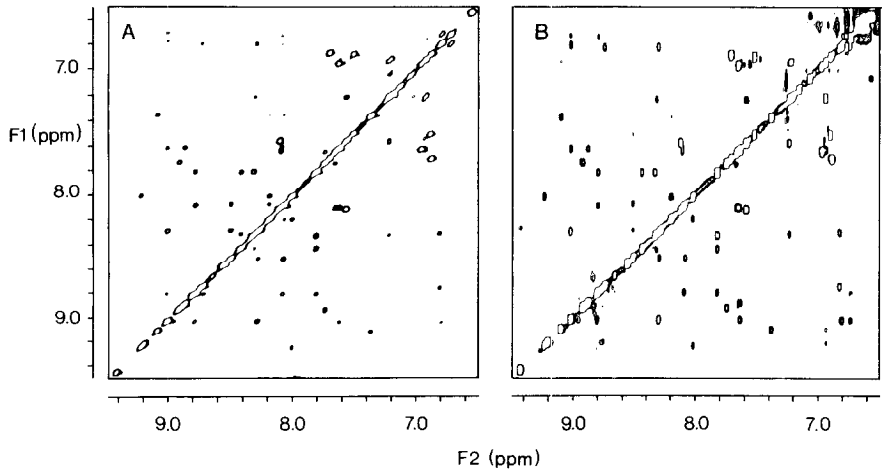

FIG. 1. (A) Soft NOESY spectrum of the NH region of 6 m*M* α_1-purothionin in 90% H_2O/10% D_2O recorded with the sequence given by Scheme [1], after baseline correction with a polynomial of sixth degree in both dimensions. [Note that the spectral width in ω_2 (2994 Hz) is larger than that displayed (1479 Hz)]. (B) The corresponding region of a hard NOESY spectrum obtained with preirradiation of the water resonance during the relaxation delay and mixing time. The experimental conditions are as follows. Soft NOESY: ω_1 spectral width = 1479 Hz, ω_2 spectral width = 2994 Hz, Gaussian 90° pulse (centered in the NH region at 8.0 ppm) = 1.6 ms, acquisition time = 0.171 s, τ_m = 0.2 s, t_1 increments = 212, transients per increment = 64. Hard NOESY: ω_1 spectral width = ω_2 spectral width = 7042 Hz, nonselective 90° pulse = 10 μs, t_1 increments = 512, transients per increment = 128. Both spectra were recorded at 25°C.

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