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Direct Identification of Relayed Nuclear Overhauser Effects

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The measurement of nuclear Overhauser effects (*1*) for determination of the three-dimensional structure of molecules in solution is rapidly gaining popularity. The buildup rate of the NOE depends on r^{-6}, where r is the interproton distance. Measurement of the NOE buildup rates therefore provides a means to measure interproton distances. Most commonly, two-dimensional NMR experiments are used for measuring the NOEs in complicated spectra of macromolecules that are in the slow tumbling limit ($\omega\tau_c > 1$). To measure the true buildup rate, the 2D experiment has to be repeated for a series of mixing times, and the cross-peak intensity is monitored as a function of this mixing time (*2–5*). A complication commonly occurs if two protons, B and C, are close in space and a third proton, A, is close to B but distant from C. In this case, a cross peak can be observed between A and C which could be mistakenly interpreted as a direct NOE connectivity between A and C. Interpretation of the intensity of this cross peak generally will lead to an erroneous AC distance determination, i.e., to an incorrect structure. In principle, the shape of the NOE buildup curve could be used to distinguish direct from relayed NOEs. However, to obtain a useful buildup curve it is especially important to measure the NOE for very short mixing times. For these short mixing times the cross peaks have very low intensities and consequently the sensitivity of this approach is poor.

It is demonstrated here that direct and relayed NOEs can readily be distinguished by using spin-locked NOE spectroscopy (*6, 7*). In the spin-locked NOE experiment the NOE effect is always positive and increases with slower molecular tumbling. Therefore, cross peaks due to direct NOE are always opposite in sign relative to the diagonal and consequently, cross peaks relayed via an intermediate nucleus will be in phase with the diagonal resonances. Of course, doubly relayed signals would be of opposite sign again but these resonances are usually too weak to be detected unless very long mixing times are used. In 1D NOE difference spectra of small molecules ($\omega\tau_c < 1$) this alternation of the sign of the NOE is commonly observed and is known as the "three-spin effect" (*1*).

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Consider as an example a proton (A) which is close to a methylene proton (B) but relatively far removed from the other methylene proton (C). Because of the short interproton distance between B and C (≈ 1.7 Å), the NOE between them builds up very rapidly. In the conventional NOE experiment a correlation between proton A and both methylene protons is generally observed unless very short mixing periods (resulting in low signal-to-noise ratios of the cross peaks) are used. In contrast, in the phase-sensitive spin-locked NOE spectrum a negative cross peak (due to direct NOE) is expected between protons A and B, and a positive cross peak (due to relay) is expected between A and C. We have calculated the cross-peak buildup in the spin-locked NOE experiment for a three-spin system where the interproton distances are: $r_{\mathrm{AB}} = 2.25$ Å; $r_{\mathrm{AC}} = 3.20$ Å; $r_{\mathrm{BC}} = 1.70$ Å; the ABC angle is 107°. The intensities for this three-spin system follow directly from solving the differential equations:

$$\frac{dM_{\mathrm{A}}(t)}{dt} = -\rho_{\mathrm{A}} M_{\mathrm{A}}(t) - \sigma_{\mathrm{AB}} M_{\mathrm{B}}(t) - \sigma_{\mathrm{AC}} M_{\mathrm{C}}(t) \qquad [1a]$$

$$\frac{dM_{\mathrm{B}}(t)}{dt} = -\rho_{\mathrm{B}} M_{\mathrm{B}}(t) - \sigma_{\mathrm{AB}} M_{\mathrm{A}}(t) - \sigma_{\mathrm{BC}} M_{\mathrm{C}}(t) \qquad [1b]$$

$$\frac{dM_{\mathrm{C}}(t)}{dt} = -\rho_{\mathrm{C}} M_{\mathrm{C}}(t) - \sigma_{\mathrm{AC}} M_{\mathrm{A}}(t) - \sigma_{\mathrm{BC}} M_{\mathrm{B}}(t) \qquad [1c]$$

Figure 1 shows the NOE buildup for A and B, starting with $M_{\mathrm{C}} = 1$ and $M_{\mathrm{A}} = M_{\mathrm{B}} = 0$. Calculations refer to the situation where $\omega\tau_c = 1.1$ and $\omega = 3.14 \times 10^9$ (500 MHz

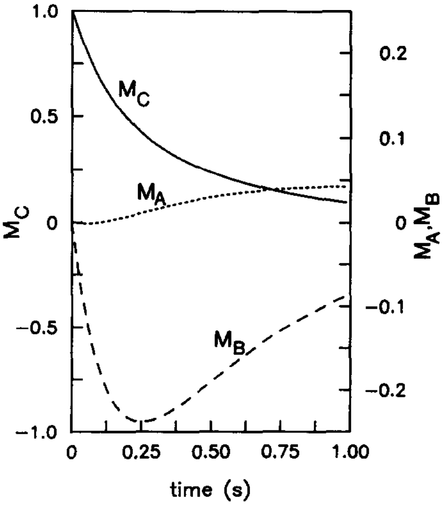

FIG. 1. Simulation of the time dependence of resonance intensities in a spin-locked NOE spectrum of three interacting spins, A, B, and C. Internuclear distances in angstroms are $r_{\mathrm{BC}} = 1.7$, $r_{\mathrm{AB}} = 2.25$, and $r_{\mathrm{AC}} = 3.2$. Calculations refer to the situation where $\omega\tau_c = 1.1$ at 500 MHz ^{1}H frequency. The decay curve of the diagonal resonance of spin C (solid line) and the buildup of the BC (broken line) and AC (dotted line) cross peaks reflect the presence of intranuclear dipolar interactions only.

^{1}H frequency). Corresponding relaxation rates in s^{-1} are $\rho_A = 0.87$, $\rho_B = 4.95$, $\rho_C = 4.26$, $\sigma_{AB} = 0.52$, $\sigma_{AC} = 0.06$, $\sigma_{BC} = 2.72$. Figure 1 shows that the BC NOE builds up very rapidly and reaches a maximum for a mixing time of about 250 ms. For very short times of the mixing period the AC cross peak is very weak and opposite to the diagonal (M_A) but for mixing times longer than 100 ms, the relay effect becomes more intense than the direct effect and the cross peak changes sign. In Fig. 1, the AC cross peak reaches a maximum for a mixing time of about 1 s; in practice proton A will interact with other protons too, resulting in a shorter value for $T_{1\rho}$, and the maximum relay intensity will then be reached at shorter mixing times.

As an example, Fig. 2 shows cross sections taken through the 2D spin-locked NOE spectrum of a 10 m*M* solution of 5′-deoxyadenosylcobalamin (coenzyme B_{12}), obtained with a 200 ms mixing time. An analysis of the complete spectrum is presented elsewhere (*8*). Figure 2a is a cross section taken parallel to the F_1 axis at the F_2 frequency of A15H′ and shows an intense NOE between A15H′ and C19H, and a very intense NOE between the geminal A15H′ and A15H″ protons. Figure 2b shows an NOE correlation between C19H and A15H″ that is in phase with the diagonal peak (A15H″) and which therefore is due to relay via A15H′. Similarly, A8H shows a direct correlation with A15H″ (Fig. 2b) and a very weak relayed interaction with A15H′ (Fig. 2a). The section taken at the F_2 frequency of C19H shows, as expected, direct correlation with A15H′ and relay to A15H″. The relay peaks are more intense than some of the weaker direct NOEs and proper identification of these relay effects is therefore essential.

As discussed before (*7*), care has to be taken when recording spin-locked NOE experiments to minimize homonuclear Hartmann-Hahn effects. Homonuclear Hartmann-Hahn magnetization transfer occurs when the difference in effective rf field strength experienced by two scalar coupled spins is not much larger than the size of the scalar coupling. It is therefore important to have a sizeable difference in effective rf field strength for the various coupled spins. This can be accomplished by using a relatively weak spin-lock field and by positioning the rf carrier away from the center of the spectrum. We choose the rf field expressed in hertz to be twice as strong as the largest offset frequency. Nevertheless, for coupled protons that resonate at nearby chemical shifts, Hartmann-Hahn effects are sometimes difficult to avoid. In this case, relay can occur via the Hartmann-Hahn mechanism and will result in a relayed cross peak that is also of opposite sign to the diagonal. This type of relay will be referred to as coherent relay. As an example of coherent relay, Fig. 3 shows an F_2 section taken through the same 2D spectrum as the sections of Fig. 2, displaying direct NOE connectivity between R1H (diagonal) and R2H. The cross peak with R3H is due to coherent relay from R2H to R3H; the distance between R1H and R3H is well over 4 Å.

We have shown that relayed NOE effects are readily identified in spin-locked NOE spectra. Of course, relayed and direct NOE effects occur simultaneously and it is possible that relay attenuates an observed direct NOE connectivity. For three spins, A, B, and C, the AC cross peak will be attenuated significantly by relay only if intense AB and BC cross peaks are present. Thus, in this case quantitative interpretation of the AC cross peak intensity should be avoided. In conventional 2D NOE experiments, relay effects constitute a major problem and have been the main reason to record a series of 2D NOE spectra for different mixing periods. For spin-locked NOE spec-

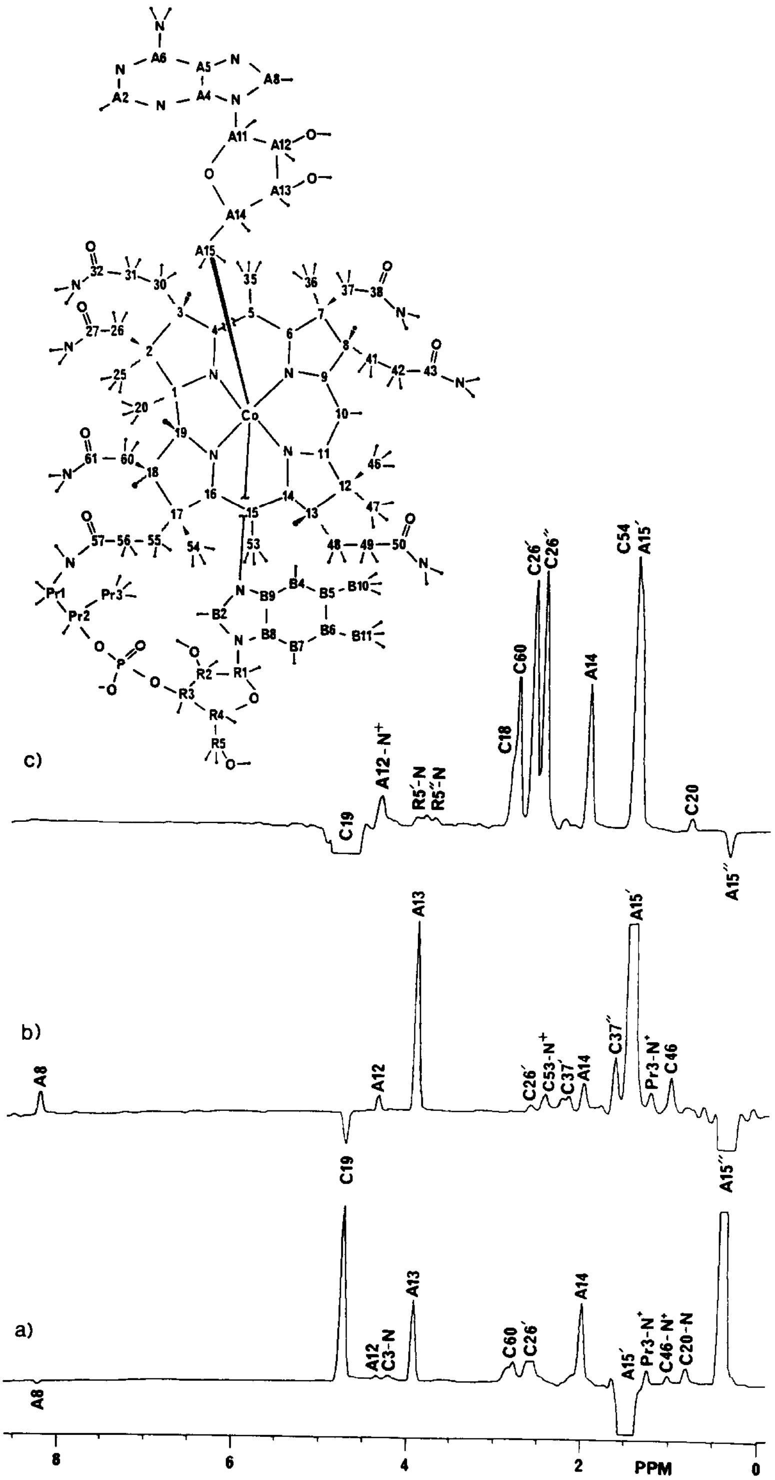

FIG. 2. Cross sections taken through the spin-locked NOE spectrum of a 10 mM solution of coenzyme B_{12} in D_2O, pH 2.1. The sections have been taken at the F_2 frequency of (a) A15H′, (b) A15H″, and (c) C19H. The diagonal and relay resonances point down; resonances due to direct NOE point up. Resonances due to baseline distortion of nearby diagonal resonances are labeled N^+; resonances labeled N represent cross peaks with a resonance that is close to the diagonal resonance.

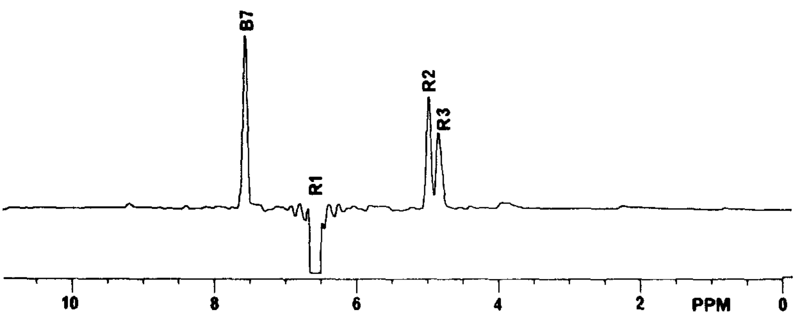

FIG. 3. F_1 cross section from the same spectrum as the sections of Fig. 2, taken at the F_2 frequency of R1H. The cross peak with R3H is due to coherent relay (via the Hartmann-Hahn mechanism) via R2H.

troscopy, however, relay effects are readily identified and semiquantitative NOE data can be obtained from a single experiment recorded with an intermediate mixing time (equal to about the $T_{1\rho}$ of the geminal protons). Of course, care has to be taken to minimize Hartmann-Hahn effects and to identify these when present (*7*). We find spin-locked NOE spectroscopy extremely useful for the study of intermediate-size molecules that exhibit near-zero regular NOE. The method is also well suited for the study of macromolecules, provided that dipolar relaxation is the main $T_{1\rho}$ relaxation mechanism.

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REFERENCES

1. J. H. NOGGLE AND R. E. SCHIRMER, "The Nuclear Overhauser Effect," Academic Press, New York, 1971.
2. A. KUMAR, G. WAGNER, R. R. ERNST, AND K. WÜTHRICH, *J. Am. Chem. Soc.* **103,** 3654 (1981).
3. R. M. SCHEEK, R. BOELENS, N. RUSSO, J. H. VAN BOOM, AND R. KAPTEIN, *J. Am. Chem. Soc.* **106,** 2914 (1984).
4. J. W. KEEPERS AND T. L. JAMES, *J. Magn. Reson.* **57,** 404 (1984).
5. U. HAHN AND H. RUTERJANS, *Eur. J. Biochem.* **152,** 481 (1985).
6. A. A. BOTHNER-BY, R. L. STEPHENS, J. T. LEE, C. D. WARREN, AND R. W. JEANLOZ, *J. Am. Chem. Soc.* **106,** 811 (1984).
7. A. BAX AND D. G. DAVIS, *J. Magn. Reson.* **63,** 207 (1985).
8. A. BAX, L. G. MARZILLI, AND M. F. SUMMERS, *J. Am. Chem. Soc.*, submitted for publication.