RELAXATION EFFECTS IN FLUORINE-LABELED PROTEINS

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NMR experiments now play an essential role in studies of the structure and function of proteins, nucleic acids, and polysaccharides. Biological macromolecules present at least two major problems to the nmr spectroscopist--their multitude of closely spaced transitions and the enhanced relaxation of these transitions due to reduced rates of molecular tumbling. An example of these effects appears in Figure 1 (top) which presents the proton nmr spectrum of hemoglobin, a protein of about 70 kDal. Although certain forms of hemoglobin have resolved single proton resonances and progress has been made in assigning these, the great bulk of the thousands of signals that make up this spectrum appear, unresolved, in the 0 to 10 ppm range. At the presently available magnetic fields, the various multi-dimensional experiments that are most useful in defining three-dimensional structure begin to falter when the macromolecule under consideration is larger than about 15 kDal molecular weight and one is forced to alternative, more specialized approaches to get even limited structural information in these cases.

Attempts to apply nmr to the study of proteins larger than the limit indicated above often involve the placement of "probe" nuclei into the structure. Isotopic labeling with carbon-13 or nitrogen-15 are examples of this approach. Another variation on this theme, namely the introduction of fluorine nuclei, can also be useful (Gerig, 1978, 1982, 1989; Sykes and Hull, 1978) because fluorine detection sensitivity is nearly the same as that of protons. While the sensitivity of fluorine chemical shielding to local environment is much higher than that of hydrogen, resolution, in the sense of shift dispersion relative to linewidths, can be good even in proteins large enough to have linewidths that would be discouraging should they appear in a proton spectrum. Figure 1 (bottom) shows the fluorine spectrum at 282 MHz of a hemoglobin into which the amino acid 4-fluorophenylalanine has been incorporated in place of phenylalanine (Gamcsik, Gerig, and Swenson, 1986). With the fluorine labeling approach used, only a certain type of amino acid appears in the spectrum, helping the assignment problem. Although the fluorine linewidths in the fluorinated hemoglobin are about 70 Hz, a resolved signal appears for virtually all of the phenylalanine positions in the protein. With the resolution and assignment problems thus ameliorated the experimenter can return to the problem of obtaining structural information.

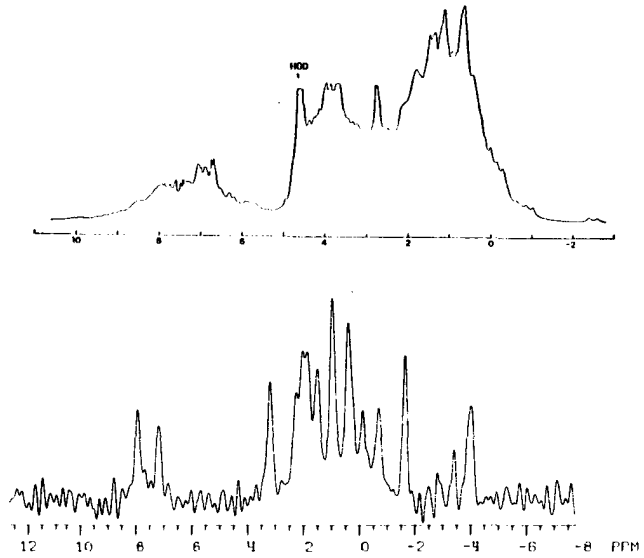
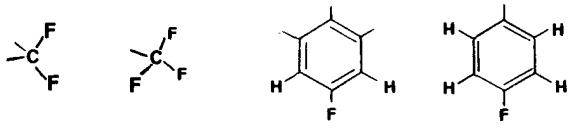


Figure 1. Top, proton nmr spectrum of hemoglobin at 500 MHz; bottom, fluorine spectrum of hemoglobin at 282 MHz.

Nmr experiments used for studies of protein structure and dynamics consist of a series of r.f. pulses interspersed with delays. When executing proton spectroscopy with large molecules, relaxation during the interpulse delays is often neglected or dealt with non-rigorously. Covalent fluorine has an appreciable chemical shift anisotropy and, although dipolar interactions provide the dominant fluorine relaxation pathways in fluorine-containing proteins, the csa contribution can be significant, especially at higher fields. Thus, consideration of relaxation effects is especially important in the design and interpretation of nmr experiments with fluorine-containing proteins. The goals of the work described here have included development of a flexible computer program which can be used to simulate the results of arbitrary multiple pulse nmr experiments and which will include a rigorous treatment of dipole-dipole and csa relaxation, including cross correlation effects, during the delays between pulses.

The program we have devised, now dubbed RELAX, is based on a complete solution of the Redfield relaxation equations (Redfield, 1965) to describe the evolution of the system density matrix during interpulse delays or the collection of a free induction decay. In it's present form RELAX uses spectral density functions appropriate for diffusive rotations of molecules that may be as complex as asymmetric tops. The effects of r.f. pulses are assumed to be instantaneous and are computed by means of rotation matrices (Aue, Bartholdi, and Ernst, 1976); the program allows phase cycling of the pulses to be investigated. The output of RELAX is designed to be directly compatible with FTNMR (Hare Research, Inc.) so that theoretical results can be worked up and plotted in the same manner as experimental data. In a straightforward implementation of the Redfield equations the dimensionality of a computational description of relaxation increases at an alarming rate as the number of spins increases. For consideration of a two spin ½ nuclei system one ends up working with 16 x 16 matrices but for a system of five spin ½ nuclei the same matrices are 1024 x 1024. In our experience, systems of 2 and 3 spins can be handled conveniently on a VAX 11/750, four spin systems are feasible under these conditions but not convenient, and systems of five spins require the capabilities of a Cray. For verification of derivations and code used in RELAX we have reproduced several relevant studies from the literature including those of Chenon, *et al.* (1982), Hull and Sykes (1975), Königsberger and Sterk (1985), Mackor and MacLean (1966), Mayne, *et al.* (1976), Stark, Vold, and Vold (1979), and Vold, Vold, and Canet (1977). These were replicated correctly, suggesting that the predictions made with RELAX are reliable.

Applications. Several fluorine-containing reporter groups that can be used in studies of proteins are shown below. Neglecting possible effects of protein protons that might be close enough to interact with these groups, they represent systems of two to five spin ½ nuclei. Some results for these spin systems obtained using RELAX will now be described. In each case the "protein systems" were represented by a sphere having a rotational diffusion coefficient corresponding to correlation time (τ_c) of 15-18 ns; these correlation times correspond to the species of 24-30 kDal that are of current interest in our lab.



I II III IV

A classical manifestation of cross-correlation effects is the introduction of asymmetry into the relative intensities of the components of complex multiplets. Using chemical shifts and coupling constants characteristic of the 4-fluorophenyl system (IV) RELAX was used to compute fluorine free induction decays following a 90° pulse. The top curve of Figure 2 shows the predicted fluorine multiplet for IV in a small molecule if relaxation is dominated by intramolecular proton-fluorine dipolar interactions and the fluorine chemical shift anisotropy but with cross correlation effects ignored. The bottom curve illustrates the asymmetry that results with inclusion of cross correlation in the calculation.

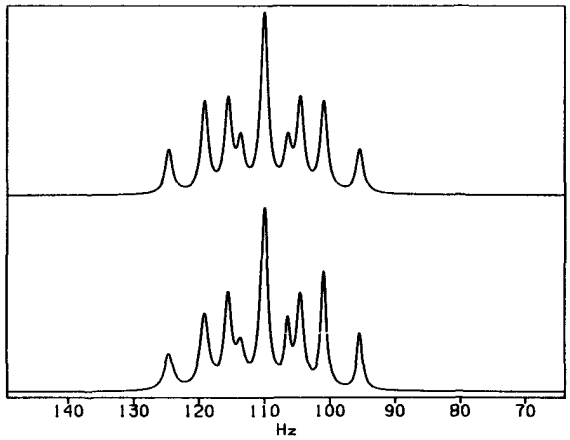


Figure 2. Predicted lineshapes for the fluorine multiplet of reporter group IV in a small molecule. Top, no dipole-csa cross correlation effects included in the relaxation equation; bottom, the results of including dipole-csa cross correlation. The fluorine resonance frequency was 470 MHz.

When the 4-fluorophenyl group is within a protein, the longer correlation time will result in increased linewidths for fluorine multiplet components so that it will not be possible to resolve the components of the multiplet. Figure 3 shows computed lineshapes for this situation. A single broad line is predicted for the fluorine spectrum. Asymmetry is still present when dipole-csa cross correlation is included but the results are more subtle now. In the presence of noise, the experimenter would likely simply mis-phase the spectrum slightly in order to produce an more Lorentzian appearance to the fluorine lineshape. Note also that the overlap of the unresolved components of this multiplet has the effect of shifting the peak maximum about 6 Hz, producing a 0.01 ppm error in the reported chemical shift at 470 MHz.

Dipolar-csa cross-correlation effects also lead to differential spin-lattice relaxation effects. Figure 4 shows the results of a simulation of a fluorine T_1 determination carried out on the three-spin model of a 4-fluorophenyl ring, III. The series of traces show the recovery of fluorine magnetization following a fluorine 180° pulse with proton-

fluorine dipolar interactions and fluorine csa being the contributors to the relaxation. If dipolar-csa cross-correlation effects are included a decided asymmetry in the spin-lattice relaxation behavior of the components of the fluorine multiplet is predicted and this, coupled with the variation in the signal linewidths predicted, leads to a distinct distortion of the signal that appears after the analyzing 90° pulse. The frequency of the peak maximum as recovery takes place varies with the recovery time. If the recovery curve were being monitored by the signal intensity at the maximum an error would be introduced. On the other hand, if the recovery process were being monitored by integrating the peak intensities, there would also be an error--notice that there is a value for the recovery time for which the lineshape would have compensating positive and negative lobes, giving a zero integral even though the magnetization has not been "nulled".

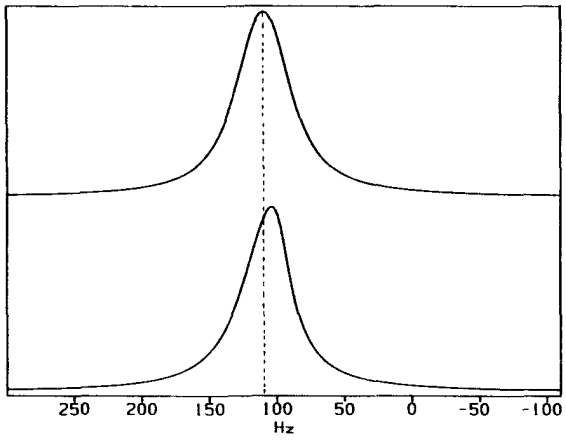


Figure 3. Predicted lineshapes for the fluorine multiplet of reporter group **IV** when in a protein. Top, no dipole-csa cross correlation effects included in the relaxation equation; bottom, the results of including dipole-csa cross correlation. The fluorine resonance frequency used in the calculations was 470 MHz.

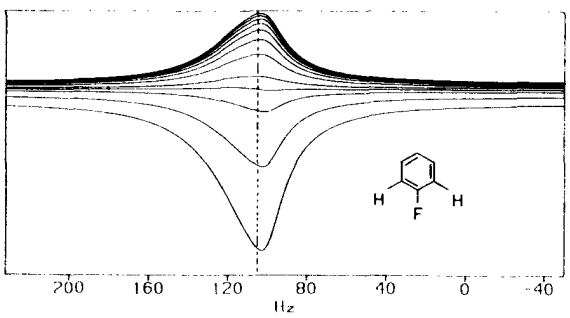


Figure 4. Simulated fluorine inversion-recovery for spin system **III** within a protein (470 MHz).

Correlation of proton and fluorine chemical shifts of those spins attached to an aromatic ring is often useful in the study of fluorine-labeled proteins. For a coupled heteronuclear correlation experiment with the three spin system **III**, one expects to find a cross-peak connecting the appropriate chemical shifts that has four components as shown in Figure 5. Note that the central transition in the triplet of features expected along the fluorine shift axis disappears because two equally intense positive and negative spectral components overlap there.

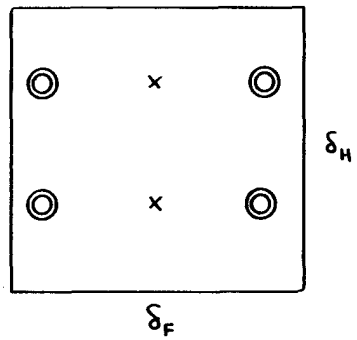


Figure 5. Schematic representation of a cross-peak formed in a coupled fluorine-proton heteronuclear chemical shift correlation experiment with reporter group **III**.

Some simulations of this experiment are given in Figure 6. The top plot of the Figure shows a predicted cross-peak with no relaxation. (The linewidths that appear are the result of modest apodization in both fluorine and proton shift dimensions.) The predicted spectrum looks like that anticipated by the discussion above. The middle plot was prepared using correlation times characteristic of our proteins and with dipole-csa cross correlation effects turned off. The lineshapes are now due to the computed relaxation behavior and it is clear that the combination of csa and dipolar mechanisms leads to a significant broadening of the fluorine resonances relative to those of the protons of the spin system. The bottom plot of Figure 6 was computed using the same correlation times as for the middle spectrum but with cross-correlation of the proton-fluorine dipolar interactions and the fluorine csa mechanism included. The asymmetry effects within the fluorine multiplet are quite pronounced, with one part of the cross-peak predicted to be considerably more intense than the other. Depending on the signal-to-noise ratio and the degree of overlap of the components of this spectrum, the fluorine chemical shift might be misjudged from the position of the observed cross-peak along the fluorine shift axis.

Similar calculations with the full five spin ½ model (**IV**) predict an even more distorted cross-peak, as shown in Figure 7. The top plot records the expected nature of a cross-peak in a coupled heteronuclear shift correlation experiment with the full 4-fluorophenyl system. These results were obtained without the inclusion of computed relaxation

effects and, again, the linebroadening in the plot was introduced by apodization. The bottom plot is the predicted cross-peak for this experiment when the full relaxation treatment, including proton-fluorine dipolar interactions, fluorine csa, and cross-correlation effects are retained. The predicted cross-peak is highly asymmetric and, in the presence of noise might produce a mis-estimate of the fluorine chemical shift. Moreover, one might altogether miss the cross-peak which correlates the fluorine shift with the shift of one of the pairs of protons in the spin system.

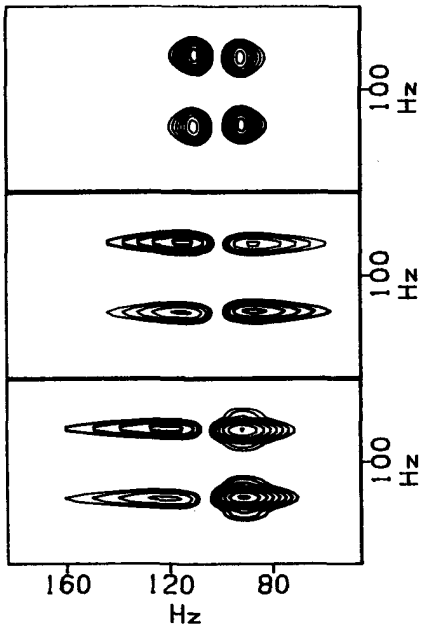


Figure 6. Simulation of the coupled fluorine-proton heteronuclear chemical shift correlation experiment at 470 MHz. Top, no explicit relaxation calculation; middle, dipolar and csa relaxation included, but without cross-correlation; bottom, as for middle plot but with the inclusion of dipole-dipole and dipole-csa cross-correlation.

One typically runs heteronuclear shift correlation experiments with decoupling in both dimensions. To achieve decoupling in f_2 certain delays are introduced into the pulse sequence. These delays are fairly long in the case of proton-fluorine correlations with fluoroaromatic rings because the corresponding coupling constants are small. Relaxation during these delays would lead to a serious loss of sensitivity and perhaps alter the predicted appearance of cross-peaks. We will be exploring optimization of this experiment with RELAX in the future.

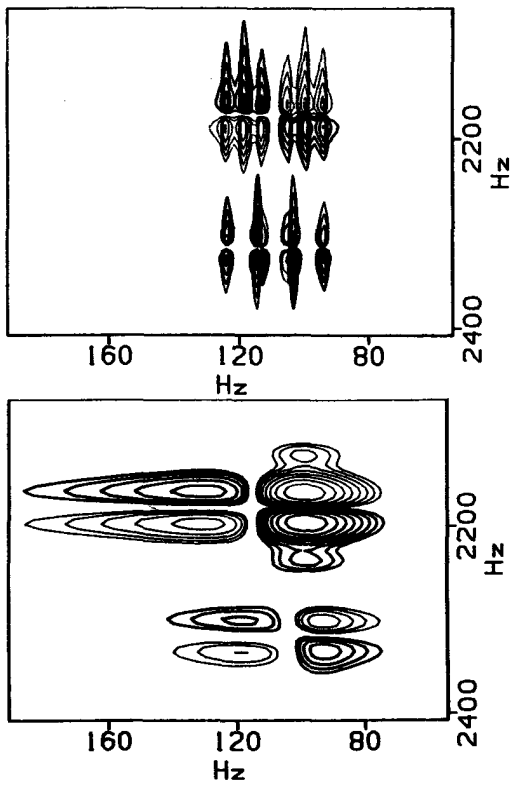


Figure 7. Computed cross-peaks for the fluorine-proton heteronuclear shift correlation experiment (coupled) for the five spin system IV. Top, no relaxation included in the calculation with some linebroadening introduced by apodization during data workup; bottom; same as for top plot but with full relaxation treatment including cross-correlations.

The CF_2 group (I) in proteins presents a two-spin ½ system which often gives an AB fluorine spectrum with J_{FF} about 250 Hz. We have investigated multiple quantum coherence generation in this system using RELAX. If the τ delay in the 2D INADEQUATE sequence (90°-τ-180°-τ-90°-t_1-90°-fid(t_2)) is set to J/4, at the end of the second 90° pulse all of the single quantum coherence is converted to double quantum coherence in an AX system (Bax, 1982). A simulation of this experiment with relaxation neglected appears in Figure 8 (top plots). If one looks closely at traces along f_1 there are small peaks at the single quantum frequencies--these presumably arise because of the non-first order nature of the spin system. Using correlation times characteristic of our proteins and including fluorine dipolar interactions and the fluorine csa contribution to relaxation, but *not* their cross correlation in a simulation of this experiment leads to the plots shown in the middle of Figure 8, with the linewidths in both dimensions now defined by the relaxation calculations. Introduction of the dipole-csa cross correlation alters the result significantly (bottom plots, Figure 8). In this case there is appreciable

asymmetry in the peak intensities and widths. Moreover, the zero quantum component as well as enhanced intensities for the single quantum frequencies are predicted. The zero quantum peak is so sharp it might be misinterpreted as artifactual in a contour plot of these results.

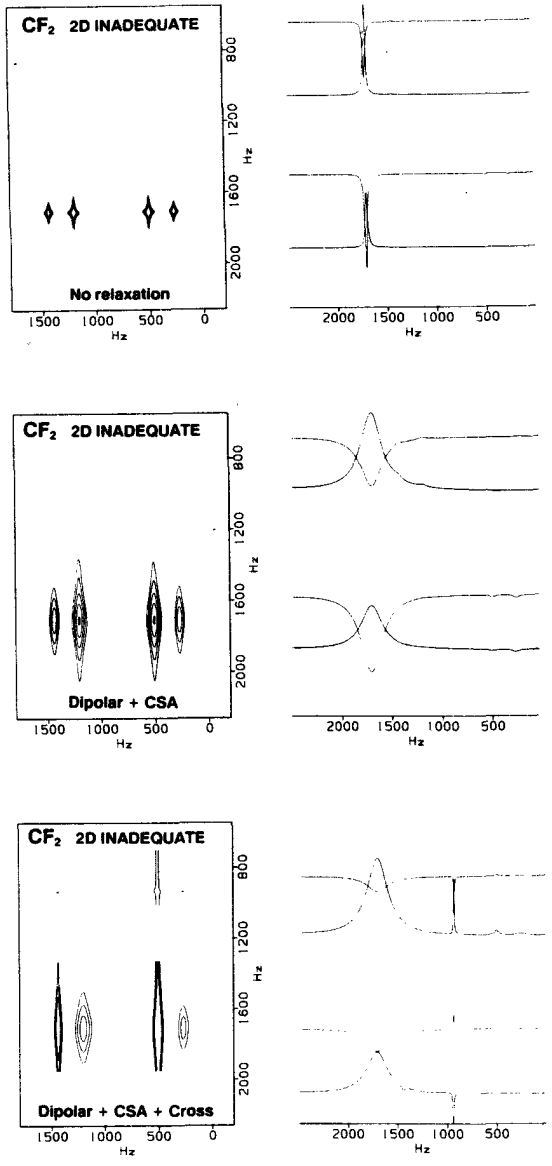


Figure 8. Simulation of the 2D INADEQUATE experiment (90°-τ-180°-τ-90°-t_1-90°-fid(t_2)) for a small molecule containing **I** assuming J_{FF}=250 Hz and the shift difference is 1000 Hz. Top plot, relaxation neglected with linewidths shown defined by the apodization used during data workup; middle plot, same spectral parameters as for the calculation presented at the top, but with correlation times characteristic of a protein and relaxation by fluorine-proton dipolar interaction and fluorine csa included without cross-correlation effects; bottom plot, same parameters as for middle plot but with cross-correlation effects included.

Müller, Ernst, Bodenhausen, and coworkers (1985, 1987) and Kay and Prestegard (1987) have discussed experiments in which relaxation effects due to dipole-dipole cross correlations lead to the formation and detection of "forbidden" multiple quantum effects. The sequence (90°-τ-180°-τ-90°-t_1-90°-90$_\varphi$-fid(t_2,ρ)) with r.f. and receiver phases cycled to produce a double quantum filtered result may be used to detect these effects. For CH_3 groups, relaxation during the τ periods and during the fid acquisition lead to an appreciable double quantum signal given certain correlation times where dipolar cross-correlation effects make spin-lattice relaxation of the methyl proton signals non-exponential. Kay and Prestegard (1987) have analyzed this experiment in detail. A calculation of the double quantum intensity as a function of the delays in the preparation period that is presented in their paper is well-reproduced by RELAX.

Under the same dynamics as used for the Kay-Prestegard calculation this double quantum effect for the CF_3 group (**II**) is predicted to be much smaller (Figure 9). The calculation represented in the bottom trace includes dipole-dipole cross correlation and csa-csa cross correlation, while the top curves includes these as well as cross correlations between the two mechanisms. Inclusion of the csa effect increases the fluorine multiple quantum relaxation rate in the CF_3 group relative to that the corresponding proton rate in the CH_3 group so that the intensity of the "forbidden" transition is decreased. Inclusion of the csa-dipole cross correlations predicts an increase in the effect of about 10-fold, into the range where it may be experimentally detectable. Utilization of this experiment in determining the dynamics of the CF_3 group in proteins is being explored further.

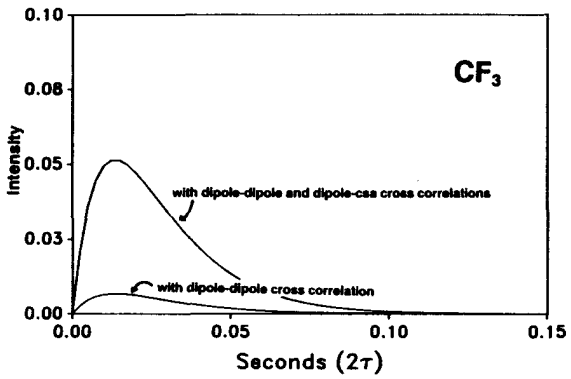


Figure 9. Simulation of "forbidden" multiple quantum experiment (90°-τ-180°-τ-90°-t_1-90°-90$_\varphi$-fid(t_2,ρ)) using the phase cycling of Kay and Prestegard (1987) for the CF_3 group.

Summary. We have produced a program for simulation of nmr experiments that takes into account the major relaxation paths for fluorine nuclei in proteins. It should be emphasized that the program is more general than the applications given above would indicate and should be usable with any spin ½ system of up to five spins.

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