JOURNAL OF MAGNETIC RESONANCE 36, 81–88 (1979)

^{19}F Longitudinal Relaxation Rates from Dynamic Negative Nuclear Overhauser Effects

S. J. OPELLA, R. A. FRIEDMAN, M. C. JAREMA, AND P. LU

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received March 8, 1979

Longitudinal relaxation rates are measured for ^{19}F under proton-decoupled conditions where the correlation time is so slow that continuous proton irradiation causes loss of magnetization due to the negative nuclear Overhauser effect. Dynamic NOE measurements yield single exponential T_1 values that are the same as those determined by inversion-recovery measurements of ^{19}F without proton-decoupling.

INTRODUCTION

There is considerable interest in ^{19}F nuclear magnetic resonance of fluorines as nuclear spin labels in specific sites of biopolymers (*1*). This is because significant simplifications of spectroscopic problems of ^{1}H NMR, such as overlapping resonances, group assignments, and dynamic range difficulties from the solvent, often result without the sensitivity limitations of ^{13}C NMR. Chemical modification of specific residues with fluorine-containing reagents or biosynthetic incorporation of amino acids or nucleotides with covalently bonded fluorines into proteins and nucleic acids can give biologically active derivatives with a manageable number of nuclear resonances from known sites.

A number of aspects of these labeled materials can be studied by ^{19}F NMR. Of course, differences among conformational states as well as binding and exchange phenomena can be monitored with the chemical shifts of the fluorines. Dynamical properties of the molecules can be obtained from ^{19}F nuclear relaxation studies; however, difficulties in sorting out the nuclear spin relaxation rates in addition to the mechanisms of ^{19}F relaxation have hindered interpretations in this area (*2, 3*). The complications from the presence of multiple competing relaxation processes are magnified by the inability to measure ^{19}F longitudinal relaxation times under ^{1}H-decoupled conditions because irradiation of the protons causes loss of fluorine magnetization.

The fluorine sites of interest on macromolecules generally have slow reorientation rates. When the protons are irradiated the resulting negative nuclear Overhauser enhancement causes a disappearance of fluorine signals. Previous T_1 measurements on fluorine-labeled proteins have been done without proton-decoupling resulting in the need to analyze the behavior of a coupled spin system which may have nonexponential behavior. An evaluation of ^{19}F T_1 measurements without ^{1}H irradiation has been made by Hull and Sykes (*2*). When considering ^{19}F spins in contact

0022-2364/79/100081-08$02.00/0

with a large number of ^1H spins at slow correlation times they concluded that T_1 determined in the absence of decoupling would be the same as in the presence of decoupling.

Experiments with slowly tumbling model systems of a single fluorine surrounded by protons and a fluorine-labeled protein presented here show that it is possible to measure the T_1 of ^{19}F with ^1H-decoupling when the equilibrium fluorine intensity is reduced to a minimum by the negative nuclear Overhauser effect. A standard gated decoupler sequence with variable length of proton irradiation prior to pulsing the fluorines yields T_1 values under decoupled conditions that can be compared to those made in the absence of decoupling. The relevant feature of this technique is that the Overhauser enhancement and consequently the observable change in magnetization develops with a rate constant of T_1.

This dynamic NOE experiment was first used by Kuhlmann and Grant for ^{13}C–H double resonance (*4*) where both T_1 and NOE could be determined for small molecules by observing the time dependence of the carbon magnetization going from $1.0M_0$ to a maximum of $3.0M_0$, where M_0 represents the normal level of magnetization at equilibrium. The slope of the semilogarithmic plot of signal intensity versus time of ^1H irradiation gives a measure of T_1 with the intercept giving the NOE. The dynamic range of this procedure is poor for carbon, particularly for slowly reorienting molecules where the enhancement is often near the minimum value of $0.15M_0$. On the other hand, the theoretical range for the corresponding ^{19}F–^1H experiment is $+1.0M_0$ to about $-0.05M_0$ and in practice is still very large.

The experimental procedure is outlined in Fig. 1. Quantitative aspects of nuclear spin interactions must be considered in carrying out the experiment and evaluating its sensitivity. The sequence is initiated by turning on ^1H irradiation for a variable time period, designated τ, which saturates the ^1H spins, removing that magnetization, and in turn causes the ^{19}F spins to lose their magnetization at their longitudinal relaxation rate because of the negative nuclear Overhauser enhancement in the systems under investigation. This is described by the equation,

$$M_{\text{observed}}(\tau) = (M_0 - M_{\text{decoupled}})\, e^{-\tau/T_1} + M_{\text{decoupled}},$$

where the observed magnetization that varies with time of irradiation is $M_{\text{observed}}(\tau)$

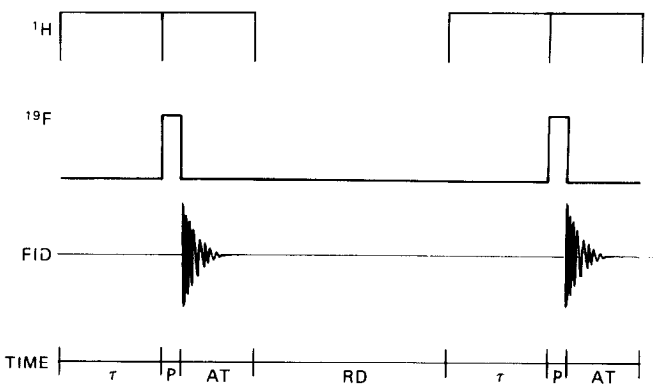

FIG. 1. Experimental procedure for determining T_1 from dynamics of NOE.

and the level of magnetization with continuous proton irradiation is designated $M_{decoupled}$. Note that $M_{decoupled} = M_0(1 + NOE)$. A 90° pulse at the ^{19}F frequency of duration P samples the reduced fluorine magnetization. The size of signal observed is a function of time τ and the rate of decrease corresponds to the single exponential function of T_1. The time interval AT represents the acquisition time for data collection; leaving the ^1H irradiation on during this period will give a decoupled fluorine spectrum. Turning off the decoupler during this interval is also consistent with determining T_1 under ^1H-decoupled conditions and may be necessary on some spectrometers to avoid noise input to the receiver, since it is often difficult to filter proton rf from weak ^{19}F signals. In fact for most ^{19}F spins with substantial negative NOE the natural linewidths are so broad that little or no increase in resolution results from proton-decoupling. The recycle delay, RD, is the time interval during which ^{19}F magnetization recovers to its equilibrium M_0 value. The time required for magnetization to recover is determined by the fluorine spin behavior while coupled to protons. In general, the same considerations discussed for ^{13}C (*3*) are relevant, resulting in a recycle delay of 8 to 10 T_1 being required to give equilibrium magnetization in the presence of nonexponential relaxation. The results shown here for a few cases indicate that fluorine relaxation in slowly reorienting systems is the same under decoupled and undecoupled conditions and follows a simple exponential course, indicating that the usual 3 to 5 T_1 of an exponential function will often be adequate to let the ^{19}F spins come to equilibrium.

EXPERIMENTAL

Three samples were used in these studies. Two were small fluorine-containing molecules dissolved in glycerol so that lowering the temperature would give slow correlation times, and one was a protein of molecular weight 150,000, the lactose repressor, where the eight tyrosines of each of the four identical subunits were replaced with 3-fluorotyrosine through biosynthetic incorporation.

The sodium salt of monofluoroacetic acid was from Sigma and dissolved in Fisher-certified reagent glycerol at a concentration of 80 mM; neither reagent was purified further and 4% by volume water was added. The 3-fluorotyrosine was synthesized from *o*-anisidine and dissolved directly in glycerol at a concentration of 160 mM. This same 3-fluorotyrosine was incorporated in the lac repressor as described by Lu *et al.* (*6*). The sample used in these experiments was native wild-type repressor 2×10^{-4} *M* in subunits in a buffer of 0.25 *M* Tris, ph 8.5, 0.2 *M* KCl, 1 m*M* EDTA, and 1.0 m*M* dithiothreitol. The protein buffer was 25% ^2H_2O and the temperature was maintained at 25°C on the protein samples.

All experiments were performed on a Nicolet NT-150 spectrometer. The magnet was an Oxford Instruments solenoid operating at 3.5*T*. Samples were run in 20-mm-o.d. tubes with about 9 ml of material. The probe used a double-tuned Helmholtz coil for ^{19}F observe and ^2H lock with a separate Helmholtz coil for ^1H-decoupling. This probe had very poor B_1 homogeneity, typical of these designs, which is the reason for the size restriction by small volume samples and the need to correct relaxation measurements for this effect. In order to keep noise from the ^1H

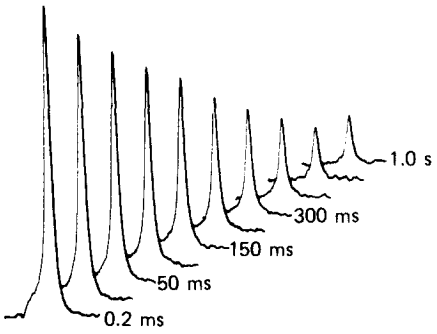

FIG. 2. ^{19}F signal from monofluoroacetic acid in glycerol at −10°C. Times near spectra are τ values from procedure in Fig. 1.

irradiation out of the ^{19}F channel, a cavity-type filter from Phelps Dodge Communications Company was employed. Pulse sequences and calculations were performed with standard Nicolet software.

RESULTS AND DISCUSSION

Monofluoroacetic acid in glycerol at −10°C is very viscous and the ^{19}F resonance is broad. The effective rotational correlation time for ^{19}F relaxation is slow enough to ensure that the magnetization with ^{1}H irradiation is at its minimum value. Figure 2 shows the effect of the gated-decoupler pulse sequence illustrated in Fig. 1 on the ^{19}F resonance signal of monofluoroacetate. The observed magnetization decreases as a result of turning on ^{1}H irradiation prior to the sampling ^{19}F radiofrequency pulse. The minimum magnetization observed under continuous ^{1}H irradiation is about +10%. The spectra from relaxation measure or continuous irradiation were not a function of decoupler power levels or modulation over a wide range of conditions.

In Fig. 3A the data of Fig. 2, along with other points not included in Fig. 2 for clarity, are least-squares fitted to a single exponential relaxation function. The fit of

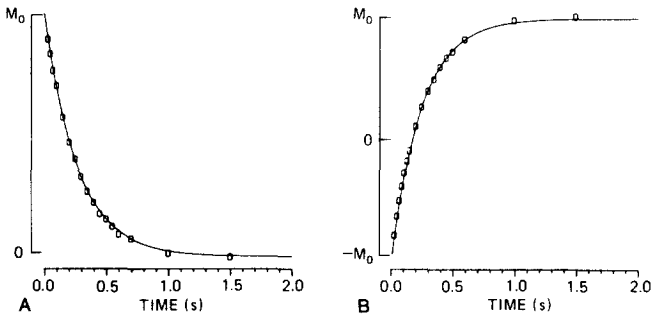

FIG. 3. (A) Least-squares fit to single exponential function data from dynamic NOE measurements on sample of monofluoroacetic acid in glycerol at −10°C. The spectrum from the continuously decoupled case was subtracted from the individual τ value spectra prior to calculating T_1. (B) Least-squares fit of inversion-recovery data on the same sample to a three-parameter equation to correct for B_1 inhomogeneity (7).

the data is excellent and a value for T_1 of 0.25 sec is derived. These data indicate that ^{19}F longitudinal relaxation rates can be determined to high accuracy and that they are single exponential functions by the dynamic NOE experiment. Figure 2B contains the data from a measurement of T_1 by inversion recovery with no ^1H irradiation fit to a three-parameter equation (*7*) to correct for effects of B_1 inhomogeneity. This fit to an exponential function is also excellent and a time of 0.27 sec was found for T_1 that is well within experimental error of the value from the gated-decoupler experiment.

Because of the high concentration of material in the fluoroacetate sample even with the ^1H decoupler turned on continuously, sufficient magnetization was observable to allow relaxation measurements. This inversion-recovery value was the same as for the nondecoupled case. The single exponential relaxation and its equivalence in rate for decoupled and coupled cases observed for these slowly reorienting ^{19}F spins relaxed by protons is distinctly different from that observed for ^{13}C relaxed by protons in rapidly tumbling small molecules (*5*). The effect of the protons forming a strongly interacting multispin system for slowly reorienting molecules clearly is having the effect predicted in the carbon case (*5*) and the fluorine case (*2*). Therefore, the recycle delay can be set to 3 to $5T_1$ with some confidence for slow correlation times and the coupled T_1 of ^{19}F is a meaningful parameter.

The same series of experiments on the glycerol solution of 3-fluorotyrosine at $-15°C$ gave similar results. This sample was used to test the multispin aspects of the Hull and Sykes treatment (*2*) where there are tyrosine ring protons of varying distances from the fluorine to be concerned with instead of the two equivalent nearby protons of monofluoroacetate. This sample is also a reasonable model for proteins with 3-fluorotyrosine incorporated.

Figure 4 contains the data from the application of the gated-decoupler sequence to lactose repressor protein with 3-fluorotyrosine substituted for tyrosine. The protein has eight tyrosines and under the conditions of pH and temperature employed here seven distinguishable resonances can be observed. Three of these resonances are broad and four are relatively sharp. The spectrum has been described previously, noting that it is readily apparent from differences in NOE, T_1, and linewidths among the fluorine resonances that there is a substantial range of fluorine relaxation behavior exhibited in the native protein (*6*). The bottom spectrum of Fig. 4 is essentially that of the decoupled protein with full NOE expressed and the top of decoupled protein without NOE. The peaks remaining in the bottom spectrum have the same intensity seen for application of continuous ^1H decoupling.

The substantial ^{19}F signal intensities remaining in the protein spectra even with continuous ^1H irradiation mean that difference spectra must be used to determine relaxation times. The subtraction of the spectrum obtained with continuous irradiation from individual partially relaxed spectra is shown in Fig. 5A. This subtraction, which costs a heavy penalty in the signal-to-noise ratio, is only necessary in the situation where the NOE is not fully negative; however, it is in these intermediate motional situations where there is doubt about justifying T_1 values determined for coupled spins, since the protons may not be coupled to each other very effectively.

For the protein data the comparison of methods is shown in Figs. 5A and B for gated decoupler and inversion recovery in the absence of proton decoupling. The values of T_1 determined from the data sets summarized in Fig. 5 are listed in Table 1.

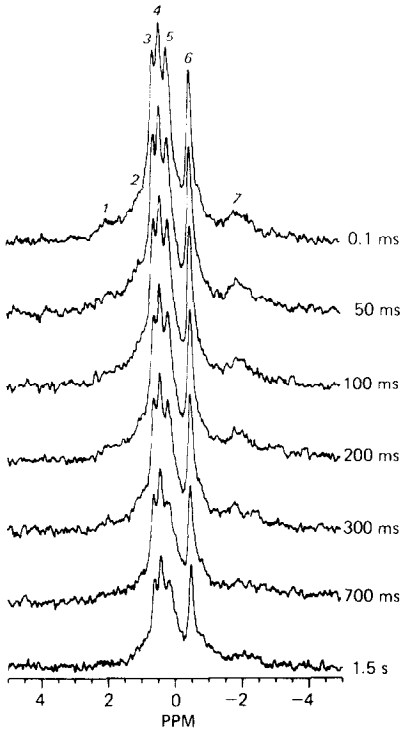

FIG. 4. Dynamic NOE data for 3-fluorotyrosine-substituted lactose repressor protein. Time of irradiation prior to the ^{19}F pulse listed near spectra. Small numbers over peaks correspond to Table 1.

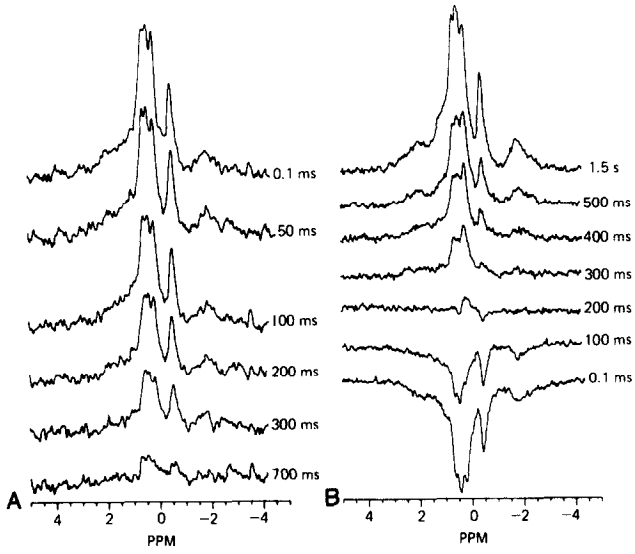

FIG. 5. (A) Spectra obtained by subtracting the 1.5-sec spectrum of Fig. 4 from the spectra with listed delay values for the dynamic NOE determination. (B) Spectra from inversion-recovery T_1 measurement on the same sample. Delays between 180° and 90° pulses listed on right near spectra.

TABLE 1

T_1 VALUES FOR TYROSINES OF LACTOSE REPRESSOR PROTEIN

Resonance	T_1 inversion recovery (msec)	T_1 dynamic NOE (msec)
1	430	
2	440	
3	400	330
4	360	350
5	330	330
6	430	440
7	400	

Within the limits of error primarily resulting from limited signal-to-noise ratios the values for ^{19}F relaxation in the presence and absence of ^{1}H irradiation are the same.

Comparison of the overall efficiencies of methods of T_1 measurement takes into account their dynamic range for observed magnetization and the time required for acquiring each free induction decay (*7*). Table 2 contains the values for the competitive methods of inversion recovery, progressive saturation, and dynamic NOE. When difference spectra are incorporated, the efficiency of the dynamic NOE method becomes very poor, although it may have to be used to ensure single exponential relaxation behavior. Progressive saturation measurement with Helmholtz coils is so error prone due to B_1 inhomogeneity that it is usually not a reasonable choice even though it is relatively efficient so the relative immunity of the dynamic NOE experiment to rf problems can be a significant factor in its use.

The T_1 values for fluorines relaxed by nearby protons can be determined conveniently with a gated decoupling procedure in the presence of ^{1}H-decoupling even when the NOE $= -1$ and there is little or no ^{19}F signal when continuous ^{1}H irradiation is applied. This allows application of straightforward relaxation theories for decoupled spin systems.

Experimentally this procedure does not suffer from the limitations of rf inhomogeneity such as inversion recovery or progressive saturation measurements. In general, the method is less efficient in terms of overall time requirements than the others, especially when it is necessary to use difference spectra because all NOEs are not at the limiting value.

TABLE 2

COMPARISON OF SENSITIVITIES OF MEASUREMENTS[a]

Method	Relative time sensitivity
Inversion recovery	1.00
Progressive saturation	1.04
Dynamic NOE	0.25
Dynamic NOE with subtraction	0.06

[a] For model experiments of 10 equally spaced τ values with equilibrium values of 3 T_1.

In test cases of small molecules in a viscous medium and a native globular protein the longitudinal relaxation of ^{19}F was found to be single exponential in nature and the same for the decoupled and nondecoupled measurements. Varying available spectrometer parameters as well as lowering the temperature until the ^{19}F signals were several kilohertz wide and near detection limits of the instrument resulted in ^{19}F signal intensities no lower than +10% in the presence of ^{1}H-decoupling relative to the normal magnetization; this is significantly different from the theoretical minimum of −5%. It may be necessary to take into account additional factors in standard relaxation theories to account for this result (*8*).

ACKNOWLEDGMENTS

This work was supported by NIH Grants to S.J.O. and P.L., and a Damon Runyon–Walter Winchell Cancer Fund postdoctoral fellowship to R.A.F. The spectrometer was obtained with a NSF grant.

REFERENCES

1. J. T. GERIG, *in* "Biological Magnetic Resonance" (L. J. Berliner and J. Reuben, Eds.), Vol. 1, p. 139, Plenum Press, N.Y. and London, 1978.
2. W. E. HULL AND B. D. SYKES, *J. Chem. Phys.* **63,** 867 (1975).
3. W. E. HULL AND B. D. SYKES, *J. Mol. Biol.* **98,** 121 (1975).
4. K. F. KUHLMANN AND D. M. GRANT, *J. Chem. Phys.* **55,** 2998 (1971).
5. S. J. OPELLA, D. J. NELSON, AND O. JARDETZKY, *J. Chem. Phys.* **64,** 2533 (1976).
6. P. LU, M. JAREMA, K. MOSSER, AND W. E. DANIEL, *Proc. Natl. Acad. Aci. U.S.A.* **73,** 3471 (1976).
7. G. C. LEVY AND I. R. PEAT, *J. Magn. Reson.* **18,** 500 (1975).
8. I. SOLOMON, *Phys. Rev.* **99,** 559 (1955).