Enhanced Sensitivity to Slow Motions Using ¹⁵N-Nitroxide Spin Labels

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The rotational motion of an ¹⁵N nitroxide in diisobutylphthalate solutions at varied temperatures has been studied for correlation times from 10^{-8} to $>10^{-3}$ sec. Data from both conventional (first-harmonic, in-phase) and saturation-transfer (second-harmonic, out-of-phase) EPR are reported. For many spectral parameters, including linewidth, position of outer extrema, and ratios of spectral amplitudes in saturation transfer spectra, the ¹⁵N-nitroxide signals are sensitive to the same range of motions as are ¹⁴N-nitroxide spectra, but the ¹⁵N data offer the convenience of enhanced signal amplitude. In addition, the peak-to-peak separation of the low-field signal of ¹⁵N nitroxides in the conventional V_1 display has a smooth dependence on rates of motion from 10⁷ to 10⁴ sec and is measured in the most intense regions of the spectrum.

INTRODUCTION

Among the more recent applications of spin labeling to studies of biological macromolecules are investigations of very large proteins or of molecular aggregates which exhibit characteristic times for rotational motion of microseconds or longer. Saturation-transfer EPR (STEPR) (1) has been developed for studying these slow rotational motions, for example, of muscle proteins (2) and of proteins rotating in membranes (3, 4). In these studies, where there is little averaging of spectral anisotropies, signal strength is often an experimental problem. In both conventional EPR and STEPR the use of ¹⁵N-nitroxide spin labels provides an enhancement of integrated signal amplitude by greater than a factor of 2 because (a) the absorption is a doublet for ¹⁵N compared to a triplet for ¹⁴N (enhancement factor of 1.5) and (b) the spread of the spectrum resulting from hyperfine anisotropy is smaller for ¹⁵N molecules than it is for ¹⁴N (enhancement factor of the order of 1.4). In addition, different m_1 regions of the ¹⁵N spectra do not overlap, as the $m_{l=0}$ and $m_{l=-1}$ regions do for ¹⁴N, so that theoretical analysis of the spectra is simplified. Accordingly, we report here the response of EPR and STEPR spectra to rates of rotational motion of ^{15}N nitroxides from 10^8 to 10^3 sec⁻¹. Theoretical computations of the effects of slow rotation on STEPR spectra of ¹⁵N nitroxides have already been made (5).

The conventional EPR spectra of ¹⁵N nitroxides were investigated previously for rotational motions ranging from 10^{-10} to 10^{-7} sec (6–9). The principal values of the g factor and hyperfine tensors for these spectra can be predicted from data for ¹⁴N spectra. Anisotropy of the g factor should not be affected by the isotopic substitution (thus $g_{zz} \approx 2.0027 \approx 2.0061 \approx 2.0089$), whereas hyperfine principal values for ¹⁵N molecules should be $\gamma_{14N}/\gamma_{15N} = 1.4$ times those of ¹⁴N molecules (giving $A_{zz} \approx 47-51$ G, $A_{xx} \approx A_{yy} \approx 9$ G).

Besides enhancement of sensitivity to slow motion, ¹⁵N nitroxides have other applications in studies of biological, or related, systems. ¹⁴N-¹⁵N nitroxide double-label experiments (9) may be used in several ways including measurement of rates of lateral diffusion in a membrane via ¹⁵N-¹⁴N spin-spin interaction (10). The sensitivity to rapid motion also renders ¹⁵N nitroxides useful for studies of intracellular viscosity (11). ELDOR studies of ¹⁴N-¹⁵N nitroxide mixtures allow effects of rotational and translational motion to be distinguished (12).

EXPERIMENTAL PROCEDURES

The spin label 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (TEMPOL) was prepared with ¹⁵N-isotopic substitution starting with the condensation of ¹⁵N ammonia (Merck, isotopic purity 95%) and acetone in the presence of calcium chloride (13). The triacetoneamine formed was oxidized to the nitroxide with hydrogen peroxide in water and reduced with lithium aluminum hydride. The product was purified by preparative thin-layer chromatography on silica gel using 2% methanol in chloroform as the solvent.

Samples for EPR were $10^{-4} M$ solutions in diisobutylphthalate. Samples were deoxygenated by bubbling a vigorous stream of nitrogen through the solution for 15 min prior to transfer to a 50- μ l disposable pipet sealed at one end. After filling, the open end of the sample tube was also sealed. Conventional EPR spectra were usually recorded at 6 mW of incident microwave power; for measurements with TEMPONE, the power was 2 mW. Saturation transfer spectra were of the V'_2 (second-harmonic, out-of-phase) display and were recorded with a modulation amplitude of 5.0 G and at a nominal 20 mW of power. Since samples were contained in the quartz Dewar insert of a Varian variable temperature attachment, the actual power at the sample was higher than the nominal value. Using the saturation of Fremy's salt for calibration, the power at the sample in the Dewar was found to be about 2.0 times the value in the absence of the Dewar insert. Figures which give power levels are in terms of the power read from the bridge multiplied by 2.0. Modulation amplitude and magnetic field were calibrated with a DPPH sample. The out-of-phase condition for V'_2 was determined by an extrapolation procedure (14).

In figures showing the dependence of spectral parameters on correlation times, τ_c , the correlation times are estimates. The estimates are based on viscosities calculated using measured temperatures and the formulas of Barlow *et al.* (15) for the temperature dependence of the viscosity of diisobutylphthalate. Correlation times were only calculated directly from the EPR lineshapes in the fast-tumbling region from 263 to 253 K. Correlation times of 12.8 and 23.4 nsec were found for 263 and 258 K, respectively. These times correspond to a volume for the rotating probe of 1×10^{-28} m³, assuming the validity of the Stokes–Einstein equation. The estimates of correlation times at lower temperatures are made from the Stokes–Einstein equation, the measured temperature, the corresponding calculated viscosity, and the above molecular volume.

In the fast-tumbling region, correlation times for ^{15}N molecules are related to spectral parameters by the formula (6)

$$\Delta \nu_{m_{I=-1/2}} - \Delta \nu_{m_{I=+1/2}} = \frac{4\tau_{\rm c}}{15\pi 3^{1/2}} b\Delta \gamma H_0$$

with

$$b\Delta\gamma = \frac{\beta_{\rm e}}{h} \bigg[g_{zz} - \frac{1}{2} (g_{xx} + g_{yy}) \bigg] \frac{4\pi}{3} \bigg[A_{zz} - \frac{1}{2} (A_{xx} + A_{yy}) \bigg] \, .$$

In using this formula, values for the g-factor anisotropy are those given in this text. Values for hyperfine anisotropy are chosen to agree with the observed separation of the outer extrema for very low temperature spectra of the diisobutyl-phthalate solutions and the observed splitting of the fast-tumbling spectra in the same solvent ($A_{zz} = 47.3$ G, $A_{xx} = A_{yy} = 9.2$ G). The peak-to-peak linewidths, $\Delta \nu$, and the magnetic field, H_0 , are in gauss.

RESULTS

Representative EPR and STEPR spectra for ¹⁵N and ¹⁴N TEMPOL are compared in Fig. 1. On the ¹⁵N STEPR spectrum (Fig. 1d) are marked amplitudes A through



FIG. 1. The EPR spectra of the ¹⁴N- and ¹⁵N-substituted nitroxide, 1-oxyl-2,2,6,6-tetramethyl-4hydroxypiperidine (TEMPOL), are compared. Absorption, first-harmonic, in-phase spectra (V_1) are shown for (a) the ¹⁴N- and (b) the ¹⁵N-substituted nitroxides. The saturation transfer (STEPR) spectra are of the absorption, second-harmonic, out-of-phase display (V'_2) for (c) ¹⁴N and (d) ¹⁵N molecules. The positions of spectral amplitudes used to quantitate the STEPR spectra are designated on the figures. For ¹⁵N nitroxides (d), the amplitudes at positions A and H are defined here as the amplitudes at the low- and high-field extrema of the spectra, respectively. All other amplitudes (B to G) are measured at a defined magnetic field increment between the position of A or H and the positions of B to G (see Table 1). For ¹⁴N nitroxides the parameters L, C, and H, and their various primes, are amplitudes above and below the baseline at spectral maxima and minima, as previously defined (17). All spectra shown are for solutions in diisobutylphthalate at 228 K.



FIG. 2. Representative STEPR spectra of an ¹⁵N nitroxide in diisobutylphthalate are shown for various temperatures.

H, which will be used here to report changes in slow rotational motion. The parameters that are most commonly used to quantitate STEPR spectra for ¹⁴N nitroxides are amplitudes at spectral maxima or minima. Although the resonance positions of amplitudes *A* through *H* fall primarily at spectral maxima or minima, some of these extrema are poorly defined at lower temperatures (see Fig. 2). A less ambiguous definition of spectral parameters is one in which amplitudes *B* through *G* are measured at constant magnetic field increments from the outer spectral maxima *A* and *H*. This procedure was used in the data presented here and these increments are given in Table 1. At temperatures greater than -40° C, the outer extrema begin to move inward. At these higher temperatures, amplitudes *A* and *H* were measured at the same field positions as in the lower temperatures spectra (i.e., at positions falling outside the outer extrema). At very low temperatures, where there are no maxima at *A* and *H*, intensities were also measured at the same, fixed magnetic fields.

Conventional EPR spectra of the first-harmonic-absorption (V_1) display have been used to measure correlation times as long as 10 μ sec (16, 17). The spectral parameters employed are (a) the inward shift of the outer extrema and (b) the outer width at half-height of the outer-extrema. As shown in Figs. 3a and b, these parameters for ¹⁵N labels can also be used to detect correlation times < 10⁻⁵ sec.

Effects of averaging of g-factor anisotropy should make the conventional EPR spectra of nitroxides sensitive to motions an order of magnitude slower than those detected by averaging of hyperfine anisotropy. Indeed, small changes in the $m_{I=0}$ line for ¹⁴N spectra are apparent in cases of slow motion where inward shifts of the outer extrema have already reached the rigid limit. The

TABLE 1

MAGNETIC FIELD INCREMENTS BETWEEN THE RESONANCE POSITIONS OF SPECTRAL AMPLITUDES A AND H AND THE POSITIONS OF $B-G^a$

Amplitude	Position
В	A – 20 G
С	A – 19 G
D	A – 25 G
Ε	A – 29 G
F	H + 11.5 G
G	H + 5.5 G

^a For temperatures from 233 to 203 K, A and H are measured at well-resolved spectral maxima. Outside this temperature range, these amplitudes are measured at fixed magnetic fields (see Results).

amplitude of the low-field line in ¹⁵N spectra has nearly the same absolute intensity as the amplitude of the middle line in ¹⁴N spectra for equivalent concentrations of label. However, the minimum of the low-field line is better defined for ¹⁵N spectra than is the minimum of the ¹⁴N $m_{I=0}$ peak so that the peak-topeak widths of the low-field ¹⁵N lines are quite useful parameters for slow motions. Clearly, from the data shown in Fig. 4, this parameter could be used conveniently for study of rates of motion from 10⁷ to 10⁴ sec⁻¹ (18).



FIG. 3a. The separation (ΔA , in gauss) of the outer maximum (low-field) and minimum (high-field) is shown as a function of correlation time for V_1 spectra of ¹⁴N (\bigcirc) and ¹⁵N (\bigcirc) nitroxides. Correlation time estimates in this and subsequent figures are based on measured temperatures and calculations of viscosity for the solvent (see Experimental section). Spectral data in this and subsequent figures were recorded at a power of 6 mW, which is a slightly saturating level.

FIG. 3b. The outer half-width at half-height of the high-field maximum ($\Delta L_{1/2}$) decreases as correlation time increases for ¹⁵N V₁ spectra.



FIG. 4. The peak-to-peak separation of the low-field signal for ¹⁵N spectra in the V_1 display is sensitive to correlation times $< 10^{-4}$ sec.

We have further checked the sensitivity of this parameter for a different nitroxide structure, TEMPONE. The peak-to-peak widths of the low-field ¹⁵N lines, or those of the ¹⁴N middle lines, have been measured for the deuteriumand proton-substituted ¹⁵N and ¹⁴N TEMPONE molecules. For ¹⁴N TEMPONE. the shape of the midfield line is such that a well-defined maximum-to-minimum width can be measured throughout the temperature range of interest. For this nitroxide, both the ¹⁵N and the ¹⁴N peak-to-peak linewidths show changes to temperatures as low as 213 K, which, using the formula of Barlow et al. (15), corresponds to a viscosity of 9.4 P and an estimated rotational correlation time for TEMPONE of 3×10^{-4} sec. The deuterium-substituted derivatives give essentially the same responses to viscosity as those of the protonated ones, derivatives, although the lineshapes differ slightly. It can be concluded, therefore, that the appropriate peak-to-peak widths are the parameters most sensitive to slow motions in the conventional V_1 displays of nitroxide spectra. Whether ¹⁵N spectra are more useful than those of ¹⁴N in this regard depends on the detailed lineshape, which varies from molecule to molecule.

Saturation transfer spectra respond to motions which are several orders of magnitude slower than those to which unsaturated spectra respond. Regions of the STEPR lineshapes where the changes in resonance position with unit angular displacement $(\partial H_{\rm res}/\partial\theta)$ are largest are the regions most sensitive to slow motion (19). Of the parameters shown in Fig. 1, the extrema at H and A are expected to be motionally insensitive $(\partial H_{\rm res}/\partial\theta = 0)$ when the conventional V_1 spectra are at the rigid limit. Indeed, Fig. 5 (upper plot) shows that the ratio H/A is nearly constant for correlation times longer than 10^{-4} sec. The amplitudes at A and H are therefore used to normalize the amplitudes measured at other spectral positions. The amplitudes at D and F are also relatively insensitive to motion. In contrast, ratios of the other parameters B, C, E, or G to A, D, or H, change markedly in the range of correlation times from 10^{-6} to 10^{-2} sec. Maximal response to slow motion varies from parameter to parameter but generally falls between 10^{-6} and 10^{-3} sec. Because of the intensity of the low-field ¹⁵N line, the parameter G/H



FIG. 5. Ratios of spectral amplitudes for V'_2 STEPR spectra of ¹⁵N-TEMPOL in diisobutylphthalate are plotted as a function of estimated rotational correlation time. The positions of amplitudes A through H are given in Fig. 1 and Table 1.

(Fig. 5) can be applied with high accuracy to studies of motion from $\sim 10^{-7}$ to $\sim 10^{-3}$ sec; on the other hand, the parameters B/A and C/A, which are measured from the high-field line, are most sensitive to slower motions.

The choice of power level and modulation in a saturation transfer experiment affects the absolute magnitude of spectral ratios. In all of our experiments, we have used a modulation amplitude of 5 G, in keeping with previous convention (19). The power used in these experiments, 20 mW at all temperatures, is the power at which the deoxygenated samples reach their maximum amplitudes at 233 K. The maximum amplitude is reached, as expected, at lower power levels for lower temperatures. In a study of slow motion with another ¹⁵N label in



FIG. 6. ¹⁵N STEPR parameters are shown as a function of microwave power for ¹⁵N-TEMPOL in diisobutylphthalate at 233 K. The power level shown is that read from the microwave bridge times 2 (see text).

another solvent, a different choice of power level may be appropriate. Also, in experiments where viscosity or motion is varied by some other means than temperature, the range of values of spectral ratios may differ from those reported here. As shown in Fig. 6, the spectral ratios reported in Fig. 5 depend strongly on the power applied to the sample (data for B/A vs power are not shown but are similar to C/A). Similar plots are obtained from ¹⁴N spectra vs power for the usual parameters C'/C, H''/H, and L''/L.

A final useful feature of ¹⁵N-nitroxide STEPR spectra is the position of the spectrum of a rapidly tumbling nitroxide superimposed on the spectrum of a slowly tumbling one (Fig. 7). This is a case frequently encountered in labeling biological macromolecules. The linear response of the conventional EPR spectra allows subtraction of the rapid-tumbling component. Therefore, the low-field peak-to-peak width shown in Fig. 4 may be obtained for the slow-moving component by subtraction. For motions slower than $\tau_c = 10^{-4}$ sec, where STEPR must be used, a rapidly tumbling spectral component interferes less with measurement of *G/H* for ¹⁵N spectra than such a component does with measurement of *C'/C* in ¹⁴N STEPR spectra.

DISCUSSION

Practical applications of spin labels to biological problems involving motions in times from 10^{-8} to 10^{-3} sec include changes in segmental flexibility of very large macromolecules, changes in rotational mobility resulting from association of components of a macromolecular aggregate, and changes in the angle between the chromophore (nitroxide) and an anisotropic-rotational-diffusion axis. For such applications it is desirable to be able to measure accurately changes in slowmotion spectral parameters which correspond to changes in correlation time of a



FIG. 7. STEPR spectra of a sample at 220 K containing slowly tumbling (diisobutylphthalate solution) and rapidly tumbling (methanol solution) populations of nitroxides. The ratio of slowly to rapidly tumbling molecules is about 5 to 1. The methanol solution was contained in a capillary inserted into the diisobutylphthalate solution.

factor of 2 or less. Several of the spectral parameters described here for the lowfield line of ¹⁵N nitroxides are particularly useful for achieving maximum sensitivity to slow motion. The relative intensities of the low-field line for ¹⁵N nitroxides compared to ¹⁴N low-field lines should be roughly 1.5 times the inverse ratio of the hyperfine anisotropies for the low-field regions of the two spectra, or $1.5 \times [21.5 \text{ G} ({}^{14}\text{N})/13.4 \text{ G} ({}^{15}\text{N})] = 2.4$. Beth et al. (20) found that perdeuteration of nitroxides gave enhanced signal amplitudes of up to 1.5 for times from 10^{-7} to $\sim 10^{-5}$ sec, which suggests that for deuterated and ¹⁵N-substituted nitroxides, a low-field signal enhancement factor of at least 3.5 over signal amplitudes for the low-field region of protonated ¹⁴N spectra should be achieved. Using the STEPR parameter G/H, there should be little difficulty in detecting a doubling of the correlation time for τ_c between 10⁻⁶ and 10⁻⁴ sec for a 10⁻⁵ M solution of ¹⁵N nitroxide. Employing deuteration and using cavities holding large sample volumes should make it possible to measure smaller changes in τ_c for a 10⁻⁵ M solution or to measure changes by a factor of 2 in τ_c within the same τ_c range for a 10⁻⁶ M solution. Measurement of even slower motions would rely on B/A or C/A parameters of the STEPR spectra. The amplitudes of the spectra in these regions are much smaller than those at G or H, so that solutions of $\sim 0.5 \times 10^{-4} M$ would be required for high accuracy.

Perhaps the most significant result of this study of ¹⁵N-nitroxide spectral parameters is that the peak-to-peak separation of the low-field line for the conventional V_1 display of ¹⁵N signals (Fig. 4) is a good parameter for motions as slow as $\tau_c = 10^{-4}$ sec. (The parameter changes by 0.5 G between 10^{-5} and 10^{-4} sec.) The intensity of this signal, and the convenience of using a linear spectral response, suggests that this parameter should have numerous applications in studies of slow motion (18).

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