A COMPARISON OF RESOLUTION-ENHANCEMENT METHODS IN SATURATION-TRANSFER EPR

^{15}N Isotopically Substituted Spin Labels and 35 GHz High-Frequency Operation

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ABSTRACT The use of ^{15}N isotopic substitution and 35 GHz (Q-band) operation as resolution enhancement methods in saturation-transfer electron paramagnetic resonance (ST-EPR) are compared. We find that both methods offer roughly comparable enhancements in ST-EPR resolution. The most powerful approach to resolving complex ST-EPR spectral behavior, however, will probably be the combined use of multiple-frequency X- and Q-band operation with ^{15}N isotopically substituted spin labels.

INTRODUCTION

The rapid passage saturation-transfer EPR (ST-EPR) techniques developed by Hyde and co-workers (1, 2) are being used to study slow molecular motions in an increasing variety of biomolecular systems. Recent applications include studies of the proteins F-actin (3), myosin (4), glyceraldehyde 3-phosphate dehydrogenase (5), lactate dehydrogenase (6) and sickle hemoglobin (7), studies of lipid behavior in model (8, 9) and erythrocyte (10, 11) membranes, and studies of such membrane protein systems as the spectrin-membrane interaction in erythrocyte membranes (11–13), the rotational behavior of rhodopsin in rod outer segment membranes (14–16) and the rotational behavior of sarcoplasmic reticulum calcium ATPase (17–20). Some of these and other applications are discussed in recent reviews (21–23).

In many of these applications the ST-EPR spectral behavior provides qualitative evidence for the existence of anisotropic motions of one sort or another. The problem of anisotropic motion in ST-EPR has been approached through the use of extensive theoretical spectral simulations (24) and through the use of experimental spin probe-thiourea adduct (25) and lipid bilayer (8) model systems. However, it has been found to be rather difficult to analyze such motion quantitatively because of the spectral overlap of spin states with the commercially available ^{14}N spin labels at X-band (9 GHz).

Two ways to enhance the resolution of ST-EPR for complex motions have recently been suggested: (*a*) increasing the observational frequency to 35 GHz (Q-band) to increase g anisotropy and spread out the spin states (26), and (*b*) substituting the ^{15}N isotope for the naturally occurring ^{14}N in the spin label nitroxide to decrease the number of spin states from three ($m_I = -1, 0, +1$) to two ($m_I = -\frac{1}{2}, +\frac{1}{2}$) (27, 28). In the present communication we describe the first comparison of these two approaches for resolution enhancement in ST-EPR. We find that 35 GHz operation with the conventional ^{14}N spin labels, and 9 GHz operation with ^{15}N nitroxide isotopically substituted spin labels both offer roughly comparable enhancements in ST-EPR resolution. Both approaches offer certain advantages: 35-GHz operation permits one to study systems using the wide variety of commercially available spin labels without the complexities of synthesizing an isotopically substituted spin label; 9 GHz operation with the ^{15}N isotopically substituted nitroxide, on the other hand, offers signal-to-noise that is approximately one order of magnitude higher than 35 GHz operation, with more convenient sample handling and instrumental operation. However, while each approach alone provides significant enhancement in resolution, the most powerful approach to resolving complex ST-EPR spectral behavior appears to be the combined use of both 9 and 35 GHz operation with the ^{15}N-substituted nitroxide labels. It appears likely that even rather complex motional behavior on the ST-EPR time scale should be interpretable through this approach.

MATERIALS AND METHODS

The ^{15}N-isotopically substituted 4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxy (Mal-6) label was synthesized following modifications of published procedures (29–32), with ^{15}N-ammonia and d$_6$-acetone as starting materials. Double integration of a spectrum of ^{15}N-Mal-6 dissolved in water indicates that the nitroxide nitrogen is substituted with >99% ^{15}N. Details of the synthesis will be published elsewhere (Bates and Currie, manuscript in preparation). Preparation and spin labeling of hemogobin (Hb) with Mal-6 followed published procedures (33, 34). Spin-labeled Hb was dissolved in various glycerol-water solutions to obtain solutions of 3% Hb with 38, 48, 87 and 92% glycerol. Ammonium sulfate-precipitated Mal-6-labeled Hb was prepared (26) for obtaining information on the highly immobilized state of Hb. Correlation times were determined as described in (26).

The conventional in-phase first harmonic (V_1) and out-of-phase second harmonic absorption ST-EPR (V_2') spectra were recorded at X-band using a Varian E-4 spectrometer (Varian Associates, Palo Alto, Calif.) and a modulation frequency of 100 kHz; a PAR model 126 lock-in amplifier (Princeton Applied Research, Princeton, N.J.) was used for phase-sensitive detection at 200 kHz for the V_2' displays. Q-band spectra were recorded on a Varian E-9 spectrometer following the procedures of Johnson and Hyde (26). Modulation amplitudes at both X- and Q-band were ~5 gauss (G) for the ^{14}N labels, and 3.5 G for the ^{15}N labels. The modulatiuon amplitude of 3.5 G was chosen since the natural linewidths of the ^{15}N-Mal-6 labeled Hb are ~3 G; modulating at the "customary" 5 G level (2) significantly over-modulates the narrow-line ^{15}N-Mal-6 spectra. X-band spectra were accumulated in a Nicolet model 1174 time averager (Nicolet Instrument Corp., Madison, Wis.); Q-band spectra were accumulated in a Tracor-Northern NS-570A time averager (Tracor-Northern Instrument Co., Middleton, Wis.). All time-averaged spectra were stored on magnetic tape for later analysis. Temperatures were measured with a digital thermometer and a copper-constantin thermocouple; accuracy of individual measurements is estimated to be ~ ± 1°C.

RESULTS AND DISCUSSION

The X-band V_1 and V_2' spectra for ^{15}N-Mal-6-labeled Hb are shown in the lower left corner of Fig. 1. The approximate positions of the $x, y,$ and z turning points for the two ^{15}N spin states ($m_I = \pm 1/2$) are shown below the V_2' spectrum. For comparison, the equivalent spectra of ^{14}N-Mal-6-labeled Hb are shown in the upper left portion of Fig. 1. It can be seen from these two sets of spectra that the isotopic substitution of ^{15}N for ^{14}N in the nitroxide results in essentially complete separation of the spin states at X-band. In contrast, in the 9 GHz ^{14}N-Mal-6 spectra it can be seen that only the $z(+1, -1)$ turning points are well resolved. The $x(+1, -1)$, $y(+1, -1)$, and all three turning points for the $m_I = 0$ spin-state overlap in the center of the spectrum for the ^{14}N-Mal-6 system. Thus, as noted by Beth et al. (27), all turning points for the two spin states of the ^{15}N-Mal-6 system are fairly well resolved.

The result of this enhanced resolution is that it should be possible to obtain much more quantitative information on the rate at which the nitroxide x- and y-axes lose correlation with the external magnetic field. The principal limitation of the ^{15}N-Mal-6 system at X-band is that, although spin-state overlap is much reduced, the four x and y turning points still lie quite close together within the spectrum. Thus distinguishing between motions which are axially symmetric with respect to the nitroxide x- or y-axes may still be fairly difficult. Anisotropic motions in which diffusional symmetry is not coincident with nitroxide magnetic symmetry will probably be even more ambiguous.

The Q-band V_1 and V_2' spectra for the conventional ^{14}N-Mal-6 labeled Hb are shown in the upper right corner of Fig. 1. From an examination of the approximate positions of the $x, y,$ and z turning points for the three ^{14}N spin states, one advantage of Q-band operation is immediately apparent: the low field peak in the V_2' spectrum is produced by the $x(+1)$ turning point, and there is enough spectral separation between it and the next higher field turning point for motional information to be obtained from the spectrum. The high field peak in this display is produced by the $z(-1)$ turning point, and there is also substantial spectral separation between it and the next lower field turning point. Thus the 35 GHz spectra should provide rather clear information on the rates of correlation loss between the external magnetic field and both of the nitroxide x- and z-axes, even for the conventional ^{14}N nitroxide labels (26).

Q-band V_1 and V_2' spectra for the ^{15}N-Mal-6-labeled Hb are also shown in the lower right portion of Fig. 1. The spectra are similar to those of ^{14}N-Mal-6 labeled Hb, with the exception that the number of turning points in the spectrum is reduced from nine to six, and the x turning

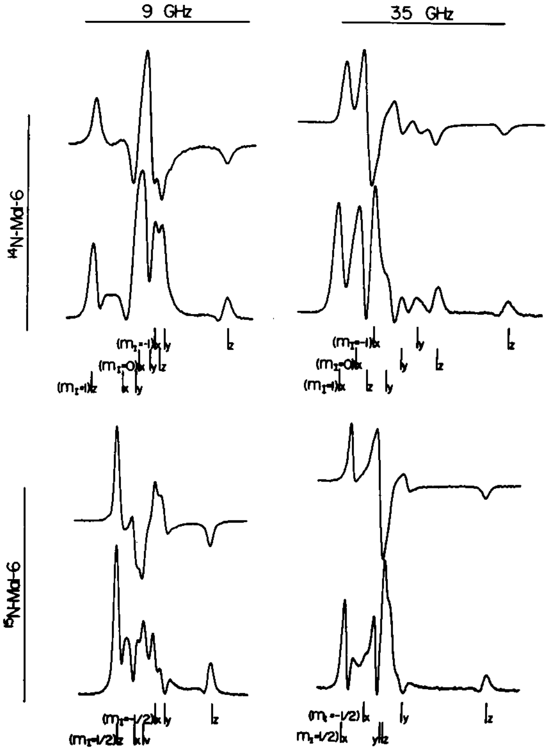

FIGURE 1 V_1 and V_2' spectra at 9 and 35 GHz. V_1 spectra are from samples of ammonium sulfate-precipitated Hb. V_2' spectra are from samples of Hb in glycerol-water solutions, with a correlation time, $\tau_R \simeq 10^{-5}$s. The spectra in the top half of the figure are for Hb labeled with the conventional ^{14}N-Mal-6. The spectra in the bottom half are for Hb labeled with the isotopically substituted ^{15}N-Mal-6. Approximate positions of the $x, y,$ and z turning points are shown below the V_2' spectra for each label and observational frequency. The V_1 spectrum is shown above the V_2' spectrum for each system.

points in the downfield region are better resolved. Thus the isotopic substitution of ^{15}N for ^{14}N results in improved resolution at both 35 and 9 GHz.

From a comparison of the four cases shown in Fig. 1, it can be seen that ^{15}N isotopic substitution at 9 GHz, and operation at 35 GHz with the conventional ^{14}N nitroxides both offer roughly comparable enhancements in spectral resolution. ^{15}N isotopic substitution at 9 GHz eliminates spin-state overlap in the center of the spectrum, but due to the close coincidence of x and y turning points, the spectra may still be ambiguous with respect to interpreting motions which selectively affect nitroxide x- or y-axis correlation with the external magnetic field; 35 GHz operation with the conventional ^{14}N nitroxides provides good resolution of both z and x turning points, but the spectra are complex, with all three spin states overlapping in the center of the spectrum.

A more powerful method for resolving complex motions than either approach alone would appear to be the use of ^{15}N isotopic substitution in conjunction with operation at both 9 and 35 GHz. Through comparisons of spectral behavior at the two frequencies, motions that selectively affect specific nitroxide axis correlation rates should be interpretable with little ambiguity.

Fig. 2 shows the spectral behavior at various rates of rotational diffusion for the V'_2 display at both 9 and 35 GHz, and for the out-of-phase dispersion (U'_1) display at 35 GHz. X-band V'_2 spectral parameters for a ^{15}N-Mal-6 system have recently been described in (27, 28) and will not be discussed here further.

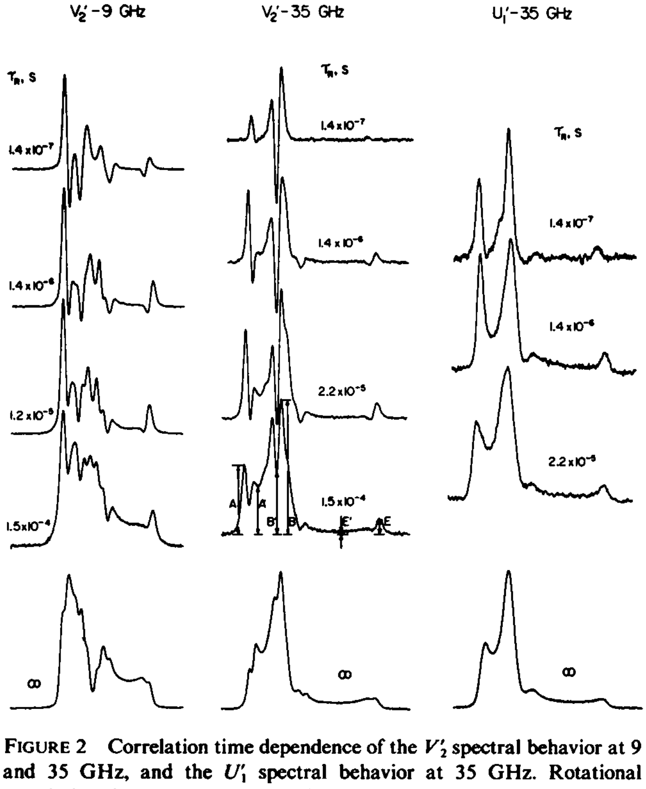

FIGURE 2 Correlation time dependence of the V'_2 spectral behavior at 9 and 35 GHz, and the U'_1 spectral behavior at 35 GHz. Rotational correlation times were calculated from the Debye equation as described in (26). The spectral parameters shown for the 35 GHz V'_2 spectra should be specifically sensitive to correlation of the x, y, and z axes with the external magnetic field, as described in the text. Labels for the spectral ratios were chosen for consistency with those previously developed for the ^{14}N-Mal-6 system at 35 GHz (26).

TABLE I

CORRELATION TIME DEPENDENCE OF 35 GHz V'_2 SPECTRAL RATIOS*

$\tau_{R(s)}$	A'/A	B'/B	E'/E
1.4×10^{-7}	0.10	−0.86	0.0
1.4×10^{-6}	0.13	−0.60	0.09
2.2×10^{-5}	0.26	−0.05	0.06
1.5×10^{-4}	0.69	+0.46	0.15
∞	1.42	+0.80	0.57

*See Fig. 2 for definitions of the spectral ratios.

Three spectral ratios, A'/A, B'/B and E'/E, which appear rather sensitive to changes in motional rates are shown for the 35 GHz V'_2 spectra in the center of Fig. 2. Numerical values for the parameters at various correlation times are given in Table I. The high field ratio, E'/E, should be primarily sensitive to loss of correlation between the nitroxide z-axis and the external magnetic field. The low field ratio, A'/A, should be primarily sensitive to loss of correlation between the nitroxide x-axis and the external magnetic field. The central ratio, B'/B, should be particularly sensitive to x, y averaging, and may be sensitive to y, z averaging. Thus the 35 GHz V'_2 spectra of the ^{15}N-Mal-6 system provide three spectral parameters which should be fairly specific to loss of correlation between the external magnetic field and each of the three principal magnetic axes of the nitroxide. Comparison of 9 and 35 GHz spectra should thus provide a route for reliable interpretation of complex motional behavior.

The 35 GHz U'_1 spectra, shown in the right side of Fig. 2, exhibit a sensitivity to motion similar to that of the V'_2 display. However the U'_1 display exhibits rather less "structure" than the V'_2 display, and the development of useful spectral parameters is somewhat more problematical. We are coming to believe that, in general, the U'_1 display will be of limited value unless used in conjunction with detailed spectral simulations.

When modulation levels are set close to the natural linewidths, we find that the conventional ^{14}N labels and the isotopically substituted ^{15}N labels provide essentially equivalent signal intensities. For a constant modulation level of 5 G Beth et al. (27) have shown that the perdeuterated ^{15}N-Mal-6 labels yield spectral intensities three to four times larger than those of the conventional ^{14}N-Mal-6 label. However, employing modulation levels in excess of the natural linewidths makes reproducibility between different spectrometers and different experimental systems rather difficult. Thus, we consider it appropriate to modulate at levels corresponding to widths of the spectral features in the systems of interest.

In summary, we find that operation with the ^{15}N-isotopically substituted nitroxide label at 9 GHz or operation with the conventional ^{14}N spin label at 35 GHz both offer comparable enhancements in spin label spectral resolution. 35 GHz operation with either the conventional or the ^{15}N label offers the advantage that turning points for two of the nitroxide principal axes (x and z) are resolved at the outer limits of the spectrum whereas the outer limits of the 9 GHz spectra are defined by the two z turning points. Signal-to-noise considerations will be of importance in considering the feasibility of 35 GHz operation, but signal-to-noise ratios are essentially equivalent for the conventional ^{14}N and the isotopically substituted ^{15}N labels when modulation levels are comparable to linewidths.

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