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^{15}N- and ^{2}H-substituted maleimide spin labels: Improved sensitivity and resolution for biological EPR studies

(glyceraldehyde-3-phosphate dehydrogenase/spin label synthesis/saturation transfer electron paramagnetic resonance/spectral simulation)

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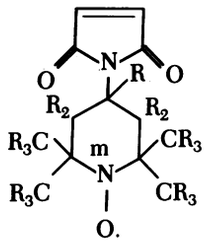
ABSTRACT **The resolution and sensitivity of electron paramagnetic resonance (EPR) and saturation transfer EPR (ST-EPR) for biological applications are greatly improved by deuteration and substitution of ^{15}N for ^{14}N in the spin-labeled probe *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL). The EPR and ST-EPR spectra of the deuterated analogue [^{2}H]MSL and the ^{15}N-substituted and deuterated derivative [^{15}N,^{2}H]MSL were compared with those of the parent MSL. The [^{15}N,^{2}H]MSL showed the greatest gain in sensitivity and the most marked sharpening of spectral features. These improvements were due to (*i*) a reduction in the spectral linewidths resulting from the relatively weak hyperfine interactions of the unpaired electron with deuterium and (*ii*) spectral simplification due to a reduction in the number of nuclear manifolds from three to two in replacing ^{14}N with ^{15}N. In the freely tumbling state, the spectra of [^{15}N,^{2}H]MSL and [^{2}H]MSL showed 10-fold and 5-fold increases, respectively, in signal heights compared to MSL. To study the slow tumbling frequencies characteristic of biological molecules, the MSL and its derivatives were covalently bound to the enzyme glyceraldehyde-3-phosphate dehydrogenase [GAPDHase; D-glyceraldehyde-3-phosphate:NAD^{+} oxidoreductase (phosphorylating), EC 1.2.1.12] on cysteine-149 of the catalytic site. The EPR and ST-EPR spectra of [^{15}N,^{2}H]MSL and [^{2}H]MSL adducts showed 3- and 1.5-fold gains in sensitivity, respectively. More important, there were striking increases in resolution, particularly for [^{15}N,^{2}H]MSL over MSL. These improvements were observed throughout the correlation time range from 0.1 μsec to 1 msec. The EPR spectrum of [^{15}N,^{2}H]MSL-GAPDHase at X-band showed no overlap of the two nuclear manifolds; therefore, all the elements of the A and g tensors could be measured directly from the spectrum. The increase in sensitivity and resolution of the ^{15}N- and deuterium-substituted spin labels permitted quantitative simulation of the EPR and ST-EPR spectra of a labeled protein. Computation time was reduced 90% by ^{15}N substitution. Use of ^{15}N-substituted and deuterated spin probes substantially improved characterization of the motional properties of a protein.**

We have shown that the sensitivity and resolution of electron paramagnetic resonance (EPR) and saturation transfer EPR (ST-EPR) spectroscopy were substantially improved for biological studies by deuteration of the frequently used maleimide spin label *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)maleimide (MSL) which binds covalently to proteins (1, 2). Significant gains in detectability and resolution were observed with [^{2}H]MSL in both the fast motion limit for freely tumbling spin label ($\tau_c \approx 10$ ρsec) and in the slow to very slow correlation time range exhibited by labeled proteins such as bovine serum albumin and hemoglobin in viscous media (0.1 μsec $\leq \tau_c \leq$ 1 msec). The improvements resulted from a reduction in the inhomogeneous line broadening due to the weaker superhyperfine interactions of the unpaired electron with deuterium than with hydrogen.

To further advance our capabilities for interpretation of spectra of labeled proteins, we prepared the doubly substituted maleimide derivative [^{15}N,^{2}H]MSL (3). It was already known



MSL, m = 14, R = H

[^{2}H]MSL, m = 14, R = ^{2}H

[^{15}N,^{2}H]MSL, m = 15, R = ^{2}H

that ^{15}N or deuterium substitution increased sensitivity for a freely tumbling spin label and that ^{15}N simplified the spectrum by reduction of the number of nuclear manifolds from three to two (4–9). However, a doubly substituted probe with ^{15}N and deuterium had not been prepared previously. The potential increase in sensitivity and resolution of such a probe seemed particularly advantageous in biological systems in which the substances to be spin labeled are often present in low concentrations and are macromolecules with slow correlation times in the range appropriate for analysis by ST-EPR techniques. The advantages of double substitution have been largely realized, as reported in this study.

MATERIALS AND METHODS

Synthesis of [^{15}N,^{2}H]MSL. The ^{15}N- and deuterium-substituted spin label was prepared by procedures similar to those for the synthesis of the compounds MSL (10) and [^{2}H]MSL (2). The deuterium composition of [^{15}N,^{2}H]MSL was determined, by mass spectral analysis, to be: 17 ^{2}H, 81.5%; 16 ^{2}H, 16.5%; and 15 ^{2}H, 2.0%. The ^{15}N- to ^{14}N-ratio of the nitroxide nitrogen was estimated, from the EPR spectrum of freely tumbling spin label, to be greater than 0.99.

Preparation and Spin Labeling of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDHase). GAPDHase [D-glyceraldehyde-3-phosphate:NAD^{+} oxidoreductase (phosphorylating), EC 1.2.1.12] was crystallized from rabbit muscle (11, 12) and spin labeled with a given maleimide analogue at a ratio of one spin label per tetramer as described by Beth *et al.* (13). GAPDHase activity was 28% inhibited by MSL and its two analogues.

Abbreviations: MSL, *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide spin label; GAPDHase, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^{+} oxidoreductase (phosphorylating), EC 1.2.1.12]; EPR, electron paramagnetic resonance; ST-EPR, saturation transfer EPR.

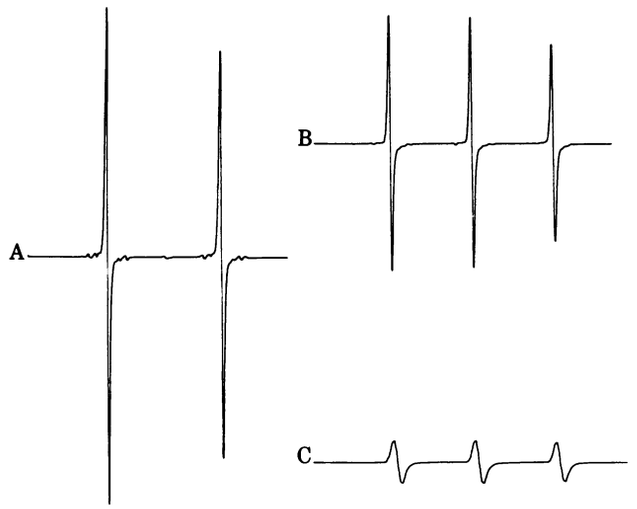


FIG. 1. X-band EPR spectra of rapidly tumbling [^{15}N,^{2}H]MSL (*A*) [^{2}H]MSL (*B*), and MSL (*C*). The spectrum of each spin label at 50 μM in 5 mM phosphate buffer (pH 8.0) was recorded with identical instrument settings at 2-mW microwave power and 0.1-G field modulation of 100-kHz frequency.

EPR and ST-EPR Measurements. EPR and ST-EPR measurements were made at 2 ± 0.5°C with a Varian E-112 spectrometer system equipped with an E-238 resonance cavity as detailed (13). Conventional in-phase absorption EPR spectra were recorded at the first harmonic of a 100-kHz field modulation of 0.5-G amplitude and 10-mW microwave power. Second harmonic out-of-phase absorption ST-EPR spectra were obtained at 50-kHz field modulation (100-kHz detection) of 5.0-G amplitude and 63-mW microwave power. Rigid limit spectra of spin-labeled GAPDHase were simulated by utilizing the computational algorithm of Balasubramanian and Dalton (14) and best fit **A** and **g** tensor values. ST-EPR spectra were calculated by using the newly developed algorithm of Robinson and Dalton (15).

RESULTS

The amplitudes of the EPR spectra of freely tumbling [^{15}N,^{2}H]MSL and [^{2}H]MSL were 10 and 5 times larger, respectively, than that of the MSL spectrum at the same label concentration and instrument settings (Fig. 1). For [^{15}N,^{2}H]MSL and [^{2}H]MSL, the enhancement in detectability is accompanied by a striking increase in the resolution of the resonance lines. The 2-fold increase in sensitivity of [^{15}N,^{2}H]MSL compared to [^{2}H]MSL is due primarily to the reduction in the number of lines from three to two and secondarily to a slightly higher overall content of deuterium in this label.

Improvements in sensitivity afforded by the [^{15}N,^{2}H]MSL label for biological spin-labeling applications are shown in Fig. 2. The EPR spectra of the [^{15}N,^{2}H]MSL- or [^{2}H]MSL-labeled enzyme (GAPDHase) in dilute buffer (τ_c = 0.17 μsec) show respectively 2.9- and 1.5-fold increases in spectral amplitude (Fig. 2 *Upper*). The same increases were observed with the enzyme in various glycerol concentrations, which gave slower tumbling rates in the range from 0.10 μsec to 1 msec. These τ_c values correspond to the motional frequencies to be expected for soluble globular proteins and their complexes in the molecular weight range from 10^5 to well over 10^8. Similar gains in sensitivity have been observed with other spin-labeled proteins, including bovine serum albumin and hemoglobin. ST-EPR spectra of the [^{15}N,^{2}H]MSL- or [^{2}H]MSL-labeled enzyme again show increased detectability (Fig. 2 *Lower*), which was observed throughout the same correlation time range from 0.1 μsec to 1 msec. The [^{15}N,^{2}H]MSL spectra were between 2 and 3 times larger than MSL over this correlation time range, and [^{2}H]MSL gave 1.3–1.8 times as much signal as MSL, depending on the correlation time.

The advantages of ^{15}N- and ^{2}H-substituted labels for detailed spectral interpretations and theoretical simulation are demonstrated in Fig. 3 *Upper Left*. Normalized EPR spectra of the

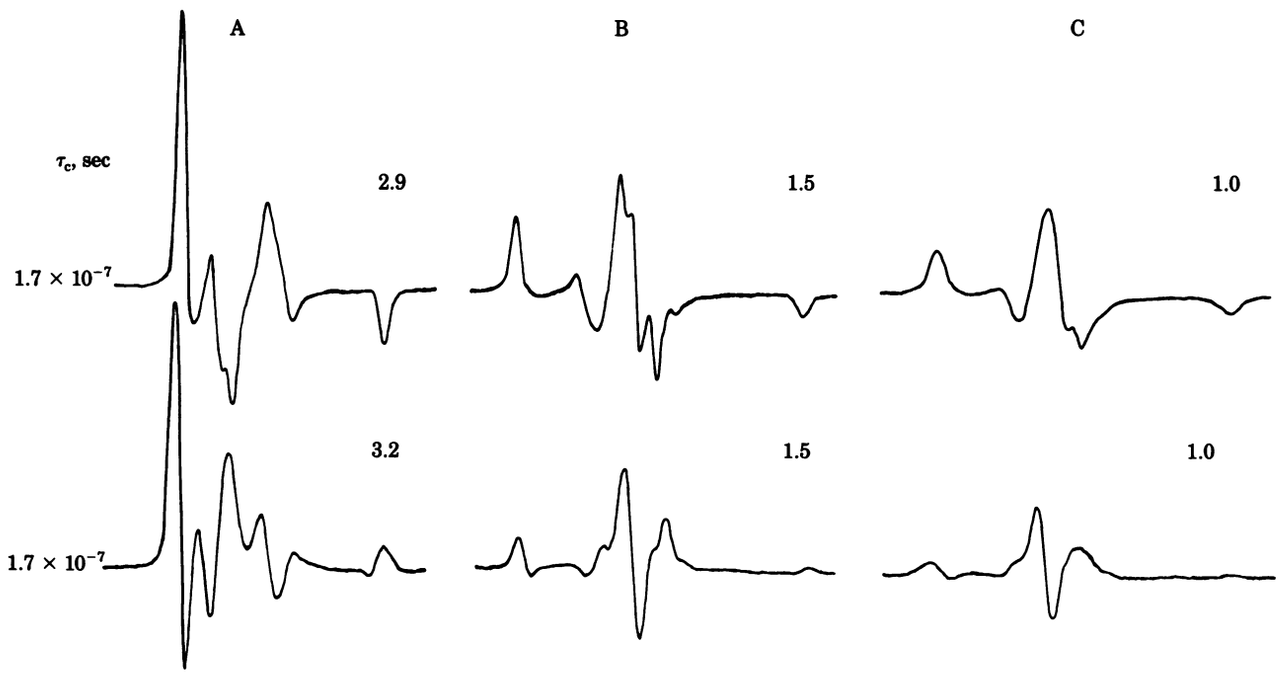


FIG. 2. X-band EPR and ST-EPR spectra of [^{15}N,^{2}H]MSL (*A*), [^{2}H]MSL (*B*), and MSL (*C*) covalently bound to GAPDHase. All spectra are 100-G displays of spin-labeled GAPDHase (50 μM) in 5 mM phosphate buffer (pH 8.0). EPR or ST-EPR spectra were recorded under identical conditions, and the sensitivity of [^{15}N,^{2}H]MSL or [^{2}H]MSL is compared to MSL taken as an intensity of 1.0.

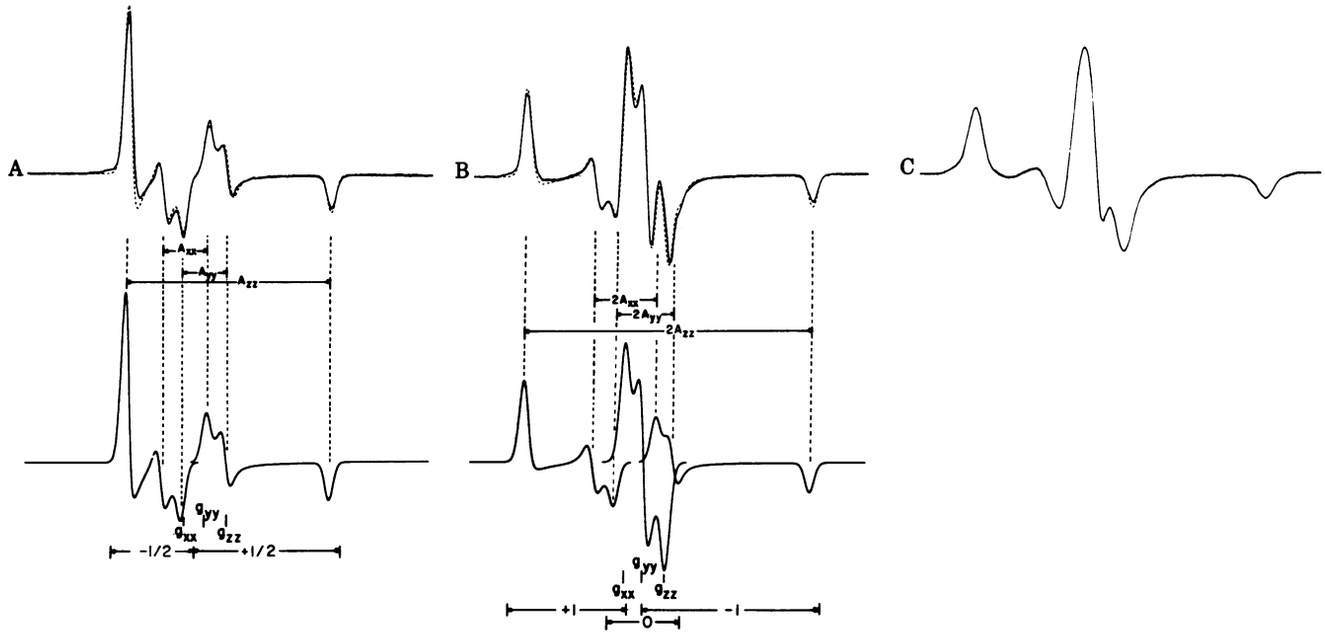


FIG. 3. Simulations of normalized EPR spectra of [^{15}N,^{2}H]MSL (*A*), [^{2}H]MSL (*B*), and MSL (*C*) bound to GAPDHase in phosphate buffer/50% glycerol (wt/vol). Experimental EPR spectra (———) were superimposed on the computer-simulated spectra (– – – – –), which were calculated using the best fit tensor values A_{xx} = 10.85 G, A_{yy} = 10.75 G, A_{zz} = 50.45 G, g_{xx} = 2.0086, g_{yy} = 2.0056, and g_{zz} = 2.0022 for the [^{15}N,^{2}H]MSL-labeled GAPDHase; A_{xx} = 7.90 G, A_{yy} = 7.40 G, A_{zz} = 35.25 G, g_{xx} = 2.0086, g_{yy} = 2.0056, and g_{zz} = 2.0022 for the [^{2}H]MSL-labeled enzyme; and the additional parameters T_{2e} = 0.2 μsec, T_{1e} = 10 μsec, and an *ad hoc* Gaussian broadening of 0.9 G for each of the simulations. Because the magnetic and microwave fields of the EPR spectrometer were not measured with sufficient precision to allow exact determination of **g** factors, the value of 2.0022 for g_{zz} was arbitrarily chosen and the other tensor values were calculated relative to it.

three MSL labels bound to GAPDHase in the rigid lattice limit show that the structuring required for accurate determination of all of the elements of the nitrogen hyperfine (**A**) and electron-Zeeman (**g**) tensors is readily observable in the [^{15}N,^{2}H]MSL spectrum. This is possible because the two nuclear manifolds of the ^{15}N label (m_I = +1/2, −1/2) are completely separated (Fig. 3 *Lower Left*). By contrast, the central portion of the [^{2}H]MSL spectrum is a complex superposition of the three nuclear states (m_I = +1, 0, −1) (Fig. 3 *Lower Right*). The overlapping manifolds in the [^{2}H]MSL spectrum prohibit accurate measurement of at least one of the minor elements, A_{yy}. For the MSL, neither the A_{yy} nor A_{xx} positions can be measured from the spectrum. The calculated lineshapes for the [^{15}N,^{2}H]MSL and [^{2}H]MSL spectra (superimposed dashed lines) are extremely good for both analogues. It should be pointed out, however, that the [^{2}H]MSL spectrum required much greater refinement and, hence, more computation time to obtain the desired accuracy than did the [^{15}N,^{2}H]MSL spectrum. This was because of the problem of overlapping spectral features and the greater number of couplings required in the calculations.

The MSL spectrum could not be satisfactorily reproduced by using the tensor values determined for the [^{2}H]MSL spectrum regardless of the linewidth used. The deuterated nitroxide label must have a slightly different ring conformation by virtue of the altered steric interactions of deuterium compared to hydrogen atoms. The simulation for the MSL spectrum therefore was not included.

The ST-EPR spectra of GAPDHase labeled with [^{15}N,^{2}H]MSL showed sensitivity to motion throughout the correlation time range from 0.1 μsec to 1 msec. (Fig. 4). We plotted the ratios L''/L and H''/H as suggested by Robinson and Dalton (15) and the additional ratio L'/L versus isotropic correlation time. These are sensitive to the rate at which the major axis of the nitroxide group (assumed to be along the nitrogen p_z orbital) is being averaged into the minor plane (the plane perpendicular to the major axis). The lineshape in the central portion of the spectrum is determined only by the structuring from the minor element interactions and therefore monitors the rate at which the x and y axes of the nitroxide group are being averaged by molecular motion.

Sensitivity to anisotropic diffusion is shown in Fig. 5. In Fig. 5*B* we simulated the experimental isotropic motion ST-EPR response shown in Fig. 5*A* at a correlation time of 20 μsec. Spectral features and relative intensities were accurately reproduced. Fig. 5*C* shows the calculated lineshape expected for an anisotropic model in which the major axis of the nitroxide label is coincident with the long axis of a nonspherical molecule (prolate ellipsoid) such as might be observed for a transmembrane protein in a bilayer. The structuring in the central portion of the spectrum was changed dramatically from the isotropic reference. With this model the x and y axes of the label were being averaged with a correlation time that was 2 orders of magnitude faster than the z and x or z and y axes were being averaged. In Fig. 5*D* we rotated the nitroxide by 90° so that the fast rotation of the molecule was averaging the z and y nitroxide axes, and a lineshape change was observed again in the center of the spectrum. In Fig. 5 *C* and *D* we chose $\tau_\parallel$ and $\tau_\perp$ so that H''/H is always the same for each spectrum. These calculations demonstrated the sensitivity of this ST-EPR response to anisotropic diffusion with ^{15}N spin labels. This approach can be used to investigate the motional properties of GAPDHase bound to the transmembrane band-3 protein, where the motion is anisotropic (16).

^{14}N spin labels also can show sensitivity to anisotropic reorientation, but analysis of ST-EPR spectra by simulation is not practical because of the excessive requirements for computer time and the complex structuring in the center of the spectra. At present, ST-EPR analyses rely on a comparison of L''/L, C'/C, and H''/H obtained from the system under investigation with

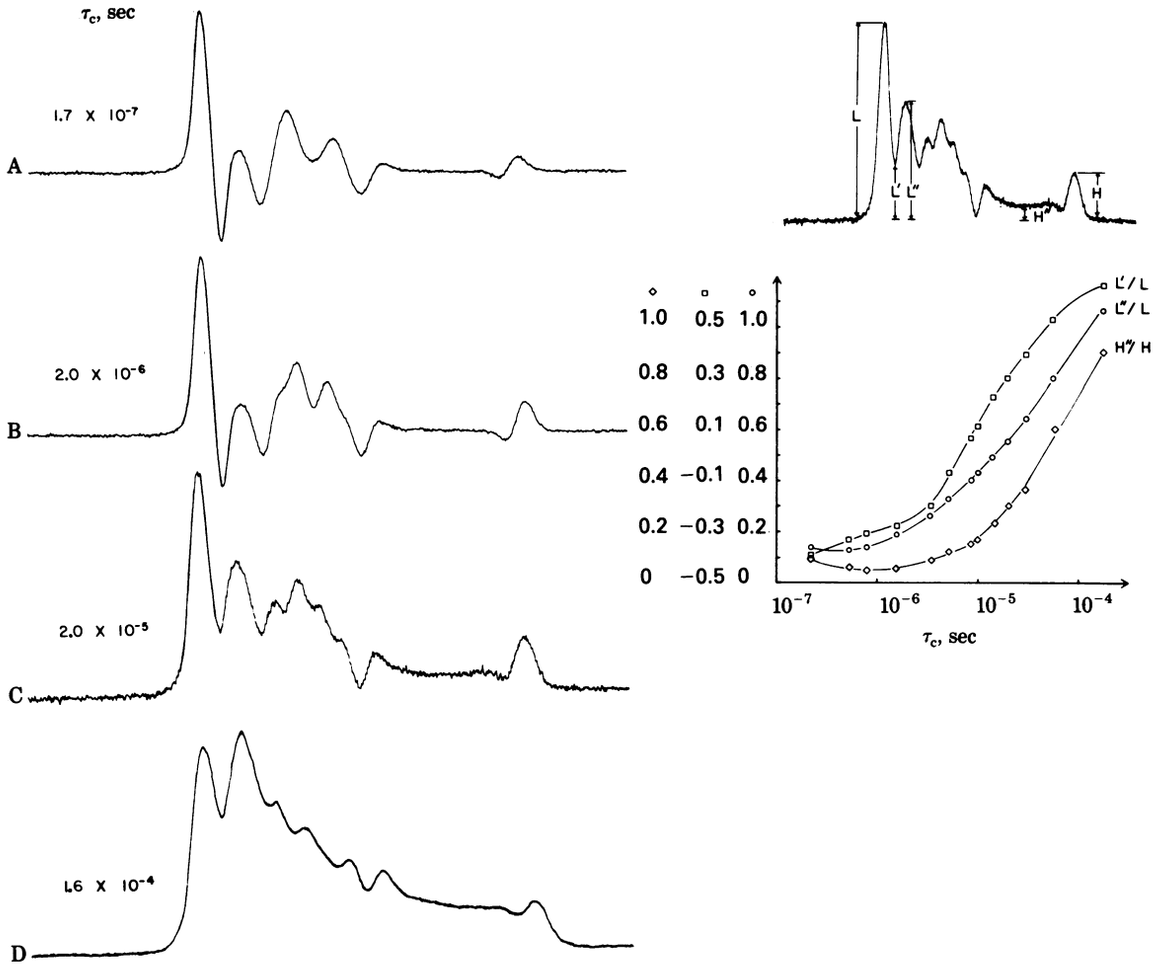


FIG. 4. ST-EPR spectra of [^{15}N,^{2}H]MSL-labeled GAPDHase in phosphate buffer containing several different concentrations of glycerol. The isotropic rotational correlation time (τ_c) was calculated with the Debye equation from the molecular radius and the measured solvent viscosities (13). Definition and plots of the ratio parameters L''/L, L'/L, and H''/H are shown on the right. The glycerol concentrations were 0%, 52%, 77%, and 90% in *A*, *B*, *C*, and *D*, respectively.

those of an isotropic model system such as spin-labeled hemoglobin (17) or GAPDHase (13). In some instances, the differences in the magnetic interactions of the system being investigated and those of hemoglobin in glycerol/water mixtures may lead to errors in interpretation. This arises because the value of C'/C is dependent on the extent of overlap of the three nuclear manifolds from ^{14}N in the central portion of the spectrum as discussed extensively by Robinson and Dalton (15).

DISCUSSION

The 3-fold increase in sensitivity of ^{15}N/deuterium labels for both EPR and ST-EPR signals over conventional ^{14}N/hydrogen spin labels is significant when the quantity of protein to be spin labeled is limiting. Detectability is also a critical factor in investigating the binding of proteins or hormones to membranes in which the concentration of receptor is low and the volume of packed membranes is limited by the dimensions of the EPR flat cell. It is possible to make EPR and ST-EPR measurements with adequate signal to noise on as little as 1 nmol (0.14 mg) of membrane-bound GAPDHase when using the [^{15}N,^{2}H]MSL label (16).

More important is the improved resolution and the level at which the data from the [^{15}N,^{2}H]MSL probe can be analyzed. Many of the approaches for determining rotational frequency or probe ordering from spectra rely upon accurate measurement of specific spectral structurings. For example, the ΔS method (18) for determining rotational correlation times requires measurement of the separation in high- and low-field extrema from EPR spectra. This can be difficult in cases in which the signal-to-noise ratio is minimal. The ^{15}N and ^{2}H probes are extremely useful in this situation because the extrema are much more highly resolved at all correlation times than they are with conventional ^{14}N/hydrogen probes.

For motional analysis by quantitative computation of experimental spectra, one must have good resolution of spectral features to optimize the agreement between experiment and theory. The ^{15}N/deuterium labels provide the necessary resolution and give the added bonus of an order of magnitude savings in computation time. The agreement between experiment and theory for both EPR and ST-EPR lineshapes using the ^{15}N/deuterium label was extremely good. The ST-EPR spectra of a biological macromolecule was quantitatively reproduced. In fact, we have chosen not to define additional parameters reflecting minor element averaging from the central portion of the ST-EPR spectrum, because direct simulation of minor element features with a motional model consistent with the system under investigation is now feasible with this label.

In summary, the results show that ^{15}N/deuterium labels significantly increase the capabilities for rigorous interpretation of data from biological spin-labeling studies. Complete characterization of motional dynamics and probe environment by

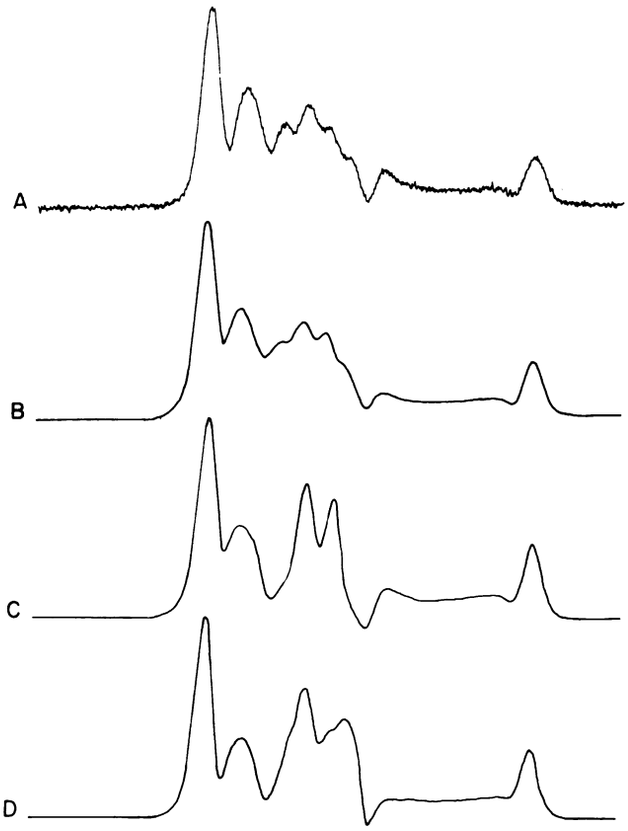


FIG. 5. Experimental and simulated ST-EPR lineshapes of ^{15}N-labeled biomolecules undergoing isotropic and anisotropic diffusion. (*A*) The experimental isotropic motion spectrum from soluble [^{15}N,^{2}H]MSL-labeled GAPDHase at a correlation time of 20 μsec. (*B*) The simulated isotropic motion spectrum calculated at the same correlation time ($\tau_\perp = \tau_\parallel = 20$ μsec). (*C*) The calculated anisotropic motion spectrum with aligned magnetic and diffusion tensors ($\theta = 0°$) and $\tau_\perp$ (20 μsec)/$\tau_\parallel$ (0.2 μsec) = 100. (*D*) The calculated anisotropic motion spectrum with orthogonal magnetic and diffusion tensors ($\theta = 90°$) and $\tau_\perp$(420 μsec)/$\tau_\parallel$ (4.2 μsec) = 100. $\tau_\parallel$ and $\tau_\perp$ are the correlation times for rotation of an asymetric molecule (prolate elipsoid) about its long and short axes, respectively. Simulation parameters included: best-fit tensor values from EPR spectrum in 77% (wt/vol) glycerol of $\mathbf{A}_{xx} = 10.625$ G, $\mathbf{A}_{yy} = 10.375$ G, $\mathbf{A}_{zz} = 50.15$ G, $\mathbf{g}_{xx} = 2.0091$, $\mathbf{g}_{yy} = 2.0061$, and $\mathbf{g}_{zz} = 2.0022$; $H_1 = 0.19$ G; $T_{1e} = 40$ μsec; $T_{2e} = 35$ nsec; and an *ad hoc* Gaussian broadening of 1.0 G.

direct simulation of experimental spectra is now more feasible than it was with conventional spin labels. It is also significant in many applications that the signal-to-noise ratio is increased by a factor of 3 with ^{15}N/deuterium labels over a wide correlation time range, facilitating studies on smaller amounts of biological material with molecular weights from 10^5 to well over 10^8.

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