REINTERPRETATION OF THE ESR SPECTRA OF MEMBRANE PROTEIN SPIN LABELS

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A number of investigators (1–5) have studied the electron spin resonance (ESR) spectra of membranes reacted with spin label analogues of *N*-ethylmaleimide. The spectrum shown in Fig. 1 *A* is for rat adipocyte membranes labeled with 2,2,6,6-tetramethylpiperidin-l-oxyl-4 maleimide (NEMSL) is typical of the type of heterogeneous spectrum generally obtained for membranes reacted with these spin labels. These spectra have been interpreted as due to a strongly immobilized component with broader bands and larger hyperfine splittings, and a weakly immobilized component with sharper bands and smaller hyperfine splittings. It is usually assumed that these two components correspond to all the spin labels reacted with the membrane and that a change in the ratio of the heights of the low field or high field bands are a direct measure of a change in the relative population of these two classes of sites (1, 3, 5).

RESULTS AND DISCUSSION

With adipocyte membranes, raising the temperature to 37°C results in an increase in the intensity of the weakly immobilized signal (Fig. 1 *B*). Furthermore, the weakly immobilized signal increases with time without any noticeable decrease in the strongly immobilized signal (Fig. 1 *B*). This time-dependent change has been shown to be irreversible and due to proteolysis of membrane proteins.[1] Irrespective of the membrane alteration responsible for the time-dependent growth of the weakly immobilized signal, these results clearly indicate that, at least for adipocytes, a fraction of the membrane spin labels are not included in either the strongly immobilized or weakly immobilized signals.

To determine whether such a fraction of spin labels is also present in other membranes, we investigated the temperature dependence of the ESR spectrum of spin labeled erythrocyte ghosts.

Fig. 2 shows that raising the temperature from 4°C to 50°C produces a nonlinear reversible increase in the weakly immobilized signal (height of the high field sharp line) without producing any decrease of the strongly immobilized signal (height of the low field broad line). Changes in the relative height of the weakly immobilized bands have previously been reported as a function of temperature (6), pH (1, 6), and protein solubilizing reagents (7). In all cases it has usually been assumed that the change in weakly immobilized signal coincides with an opposite change in the strongly immobilized signal. We have, however, been able to show that the increase in temperature from 4°C to 37°C corresponds to a ~ 30% increase in the total number of detectable spins as determined by double integration of the first derivative spectrum.

By investigating the effect of sulfhydryl cross-linking reagents (8) on this temperature dependence we have shown that spin labels reacted with pairs of free sulfhydryls close enough to form disulfide bonds are responsible for these undetected spins (9).

The temperature dependent intensity of these spin labels located close to each other can be explained by dipolar broadening of these signals. As previously shown for the interaction of paramagnetic metal ions with spin labels (10, 11), this dipolar interaction can result in a decrease in the signal intensity which depends on the distance between the spins without any noticeable broadening of the observable signals.

These studies indicate that protein spin-label spectra involve at least three classes of spin-labeled sites, and cannot be analyzed simply in terms of the ratio of peak

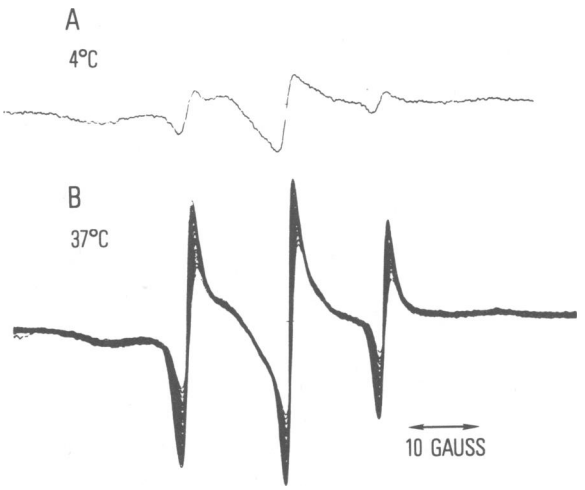

FIGURE 1 ESR spectra of rat adipocytes membranes labeled with NEMSL. After labeling the membranes were washed until no ESR signal was detected in the supernatant. Spectra were then obtained on a Jeolco (Tokyo, Japan) JES ME-IX ESR spectrometer with a variable temperature accessory. *A*, 4°C; single 10-min scans; gain = 500; response = 1 s; *B*, 37°C; repetitive 1-min scans; gain = 500; response = 0.3 s. The initial spectrum has the lowest intensity. The final highest intensity spectrum was obtained after 50 min.

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[1]Rifkind, J. M., J. T. Wang, and G. S. Roth. Unpublished results.

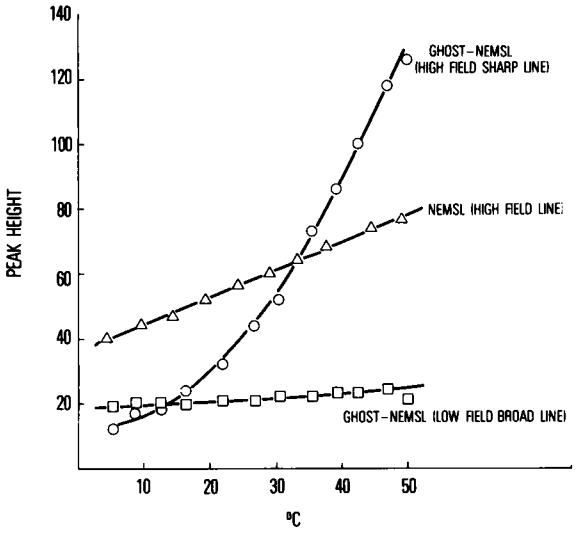

FIGURE 2 Variation of peak heights with temperature for erythrocyte ghosts labeled with NEMSL and free spin label. The instrumentation and techniques are the same as described in the legend to Fig. 1.

heights at any one set of conditions. Furthermore, the inverse r^6-dependence of the decrease (10) in signal intensity due to dipolar interactions (10) and the fact that most of these pairs of sulfhydryls are generally at the contacts between protein molecules (8, 12) provide a new, very sensitive, probe for changes in the distance and/or orientation of certain membrane proteins relative to each other.

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ADVANTAGES AND LIMITATIONS OF SPIN LABELING IN QUANTITATING PROTEIN-LIPID ASSOCIATIONS

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A central problem in lipid-protein interactions is how the hydrophobic regions of the irregular protein surface are interfaced with the fluid lipid bilayer of the membrane. The proteins are well-defined three-dimensional structures around which the lipids must conform in a way that leads to a minimum energy configuration of the system. Interesting aspects of this problem include segmental motion of the lipids in contact with the protein, conformation and orientation of these lipid chains solvating the protein, kinetics of the exchange between these lipids and the bulk bilayer solvent, and the relative binding constants of different kinds of phospholipids. The sensitivity and short time scale of ESR and the fact that spectral line shapes are sensitive to motion has made this one of the most useful spectroscopic probes for studying lipid-lipid and lipid-protein interactions. Although this field is relatively new and there is still some controversy, the current picture is that most, if not all, lipids in contact with the protein exhibit reduced motion compared with lipids in the bilayer (1). The lipid is somewhat spatially disordered by the irregular protein surface, and this lipid exchanges with bulk bilayer. (This exchange is evidently rapid on the

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