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PROPERTIES OF THE STRONGLY IMMOBILIZED SIGNAL OBSERVED IN SPIN-LABELED ERYTHROCYTES

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A strongly immobilized signal from fatty acid spin labels was observed in human erythrocytes treated with oxidizing agents such as glutaraldehyde, hydrogen peroxide, phenylhydrazine and copper-*ortho*-phenanthroline. This signal was also observed in freshly prepared ghosts treated with potassium superoxide and in old erythrocyte ghosts. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these samples demonstrated the diffuse, nondiscrete bands of high molecular weight due to the cross-linking of membrane proteins. The temperature and pH dependences of the outer hyperfine splitting of this signal were very similar to those of bovine serum albumin. We propose that the strongly immobilized signal reflects the interaction of the lipids with the cross-linked products of membrane proteins.

Introduction

Fatty acid spin labels have been widely used to measure the membrane fluidity in model and biological membranes [1]. The dynamic states of membrane, extended from its surface to the interior, can be monitored using the spin labels which bear a nitroxide radical attached to different positions along the hydrocarbon chain [2]. Such spin labels display the distinct response for external perturbations, i.e., temperature [3,4], pH [5,6], added cholesterol [6–8], etc.

For certain fatty acid spin labels, two different signals represented by the weakly immobilized label and the strongly immobilized label are observed simultaneously in biological membranes such as erythrocytes [3,4]. Up to the present time, the weakly immobilized signal has been utilized as the main information obtained from these spin labels, whereas the property of the strongly immobilized component has not yet been elucidated as the former. The strongly immobilized signal appears only in membranes with low lipid to protein ratios, but not in lipid vesicles prepared from biomembranes. Therefore, it has been considered that the strongly immobilized component is due to such lipid-protein interactions as characterized by the existence of boundary lipids [9,10].

Recently, Watts et al. [11,12] have demonstrated that such an immobilized component is also observed by the extensive bleaching or delipidation of the rod outer segment disk membranes. From the temperature dependence of this component, they have revealed that both strongly immobilized signals arise from quite different origin, and that the signal appeared by the delipidation or bleaching is attributed to the lipids trapped within aggregated proteins.

We report the properties of the strongly im-

Abbreviations: 5-nitroxide stearic acid, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl; 12-nitroxide stearic acid, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl; 16-nitroxide stearic acid, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl; SDS, sodium dodecyl sulfate; phosphate-buffered saline, 0.15 M NaCl/10 mM sodium phosphate buffer.

mobilized signal observed in human erythrocytes under various conditions.

Materials and Methods

Materials. Three kinds of fatty acid spin labels, 2-(3-carboxypropyl)-2-tridecyl-, 2-(10-carboxydecyl)-2-hexyl- and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as 5-nitroxide stearic acid, 12-nitroxide stearic acid and 16-nitroxide stearic acid, respectively) were purchased from Syva Co. Bovine serum albumin was obtained from Armour Pharmaceutical Co. Other chemicals were of analytical grade.

Preparation of intact erythrocytes and ghosts. Heparinized blood from healthy adult donors was centrifuged and plasma and buffy coat were removed. Erythrocyte ghosts were prepared according to Dodge et al. [13]. Red ghosts containing different amounts of hemoglobin were prepared as follows. Intact erythrocytes were hemolysed by changing the volume ratio of cells to buffer (5 mM sodium phosphate, pH 8) at 0°C. The hemolysate was made isotonic by adding 3 M NaCl and then incubated to reseal the open ghosts for 1 h at 37°C. The red ghosts were washed three times with 0.15 M NaCl/10 mM sodium phosphate buffer (pH 7.4). The pH of phosphate-buffered saline was 7.4 unless otherwise noted. Ghosts stored beyond a half year at −10°C, thaw a few times in the interval, are here referred to as old ghosts.

Preparation of hemoglobin-entrapped liposomes. Human hemoglobin was obtained according to the method of Fung [14]. In order to entrap hemoglobin within liposomes, a thin layer was prepared in the test tube by lipid extraction [15] from erythrocytes (pellet 3 ml), to which 1 ml of aqueous hemoglobin solution was added. The solution was shaked on a Vortex mixer with glass beads and stood in an ice bath for 3 h. The multicompartment liposomes containing hemoglobin were washed three times with phosphate-buffered saline. The concentration of hemoglobin was determined by alkaline hematin [16] or cyanomethemoglobin method [17]. The latter was used in the low concentration of hemoglobin. In order to normalize the hemoglobin content within membranes, the concentration of phosphate or cholesterol was determined by the method of Ames [18] or Zlatkis et al. [19], respectively.

Reactions of oxidants with erythrocytes, ghosts and hemoglobin-entrapped liposomes. The oxidative damages of erythrocytes, ghosts and hemoglobin-entrapped liposomes were caused by means of oxidants such as glutaraldehyde, hydrogen peroxide, phenylhydrazine, potassium superoxide and Cu-*o*-phenanthroline. The reactions of hydrogen peroxide with erythrocytes, ghosts and hemoglobin-entrapped liposomes were carried out in phosphate-buffered saline containing 4 mM NaN_3. In the case of Cu-*o*-phenanthroline, these membranes were treated with 5 mM cupric sulfate and 25 mM *o*-phenanthroline in phosphate-buffered saline. In the reaction of ghosts with potassium superoxide, ghosts (pellet 100 μl) were suspended in 4 ml of phosphate-buffered saline. The solution of potassium superoxide (7 mg) in 0.1 ml of dimethyl sulfoxide was added to ghost suspension. In all cases, the reaction mixtures containing both oxidizing agents and about 100 μl of erythrocytes, ghosts or liposomes in 4 ml of phosphate-buffered saline were incubated for 1 h at 37°C.

pH dependence. The effect of pH on membrane fluidity of erythrocytes was examined in the following buffers: 0.15 M NaCl/10 mM sodium acetate, pH 5.0; 0.15 M NaCl/10 mM sodium phosphate, pH 6.5 and 7.4; 0.15 M NaCl/10 mM Tris-HCl, pH 9.1. In each buffer, the oxidant-treated erythrocytes were incubated for 30 min at 0°C and then spin-labeled. The pH dependence of ESR spectra in fatty acid spin-labeled proteins was examined using 5%(w/v) albumin solution prepared with the buffers mentioned above.

Spin labeling and electrophoresis. Spin labeling of membranes and bovine serum albumin was carried out as described previously [4]. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of membrane proteins and the nomenclature of the polypeptide bands were performed according to Fairbanks et al. [20].

ESR measurement. ESR measurements were carried out on a JEOL JES FE-1X spectrometer (9.2 GHz) equipped with a variable temperature accessory. The temperature control was checked with a copper-constantan thermocouple and found to be stable within ±0.2 deg. C. The samples were packed into a hematocrit capillary tube. The usual spectrometer settings were 100 kHz modulation amplitude, 1 gauss; microwave power, 20 mW; scan range, 200 gauss; scan speed, 8 min.

Results

Fig. 1 shows the ESR spectra of 12-nitroxide stearic acid incorporated into glutaraldehyde (0.25 M)-treated erythrocytes and ghosts. In the case of white ghosts, the weakly immobilized component was observed predominantly (Fig. 1a). With increasing the hemoglobin content within the membranes, i.e., in red ghosts, both the strongly immobilized component and the weakly immobilized one appeared simultaneously (Fig. 1b). In erythrocytes, the strongly immobilized component alone was observed (Fig. 1c). Such a strongly immobilized component was also observed by the reactions of erythrocytes with oxidants such as hydrogen peroxide (1–20 mM), phenylhydrazine (5 mM) and Cu-*o*-phenanthroline, and its component appeared obviously with increasing concentration of oxidants. On the other hand, in the case of ghosts, the strongly immobilized signal was observed in old ghosts in which hemoglobin was free and also in fresh ghosts treated with potassium superoxide (25 mM), but not dominantly with other oxidizing agents. To examine in detail the membrane state in the oxidation of erythrocytes, 5-nitroxide stearic acid and 16-nitroxide stearic acid were used. In the reactions of erythrocytes with oxidants, both spin labels also demonstrated such strongly immobilized signal but the appearance of its component was not so dramatic as 12-nitroxide stearic acid. Therefore, 12-nitroxide stearic acid was used unless otherwise noted.

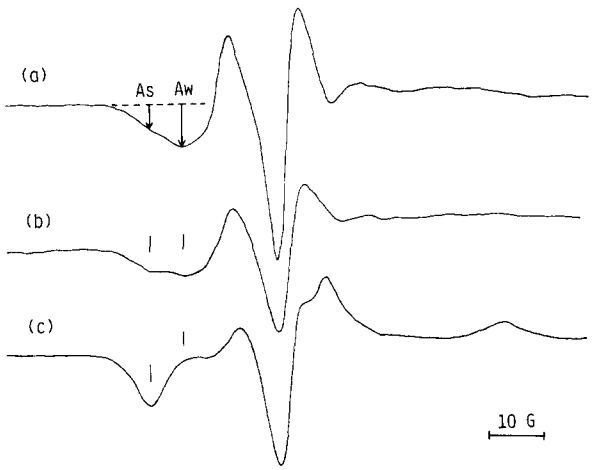

Fig. 1. ESR spectra of 12-nitroxide stearic acid incorporated into glutaraldehyde-treated white ghosts (a), red ghosts (b) and erythrocytes (c). Each membrane was incubated in phosphate-buffered saline containing 0.25 M glutaraldehyde for 1 h at 37°C. The ESR spectra were measured at 20°C. 'As' and 'Aw' indicate the amplitudes of the strongly immobilized component and the weakly immobilized component measured from the horizontal base line, respectively.

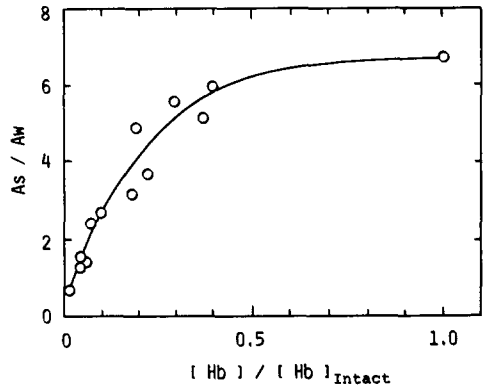

Fig. 2. Variation of As/Aw for 12-nitroxide stearic acid vs. hemoglobin content within membranes. The hemoglobin content, [Hb], is normalized by the concentration of hemoglobin contained within intact erythrocyte, $[Hb]_{Intact}$. Red ghosts were prepared as described under Materials and Methods and were treated with 0.25 M glutaraldehyde for 1 h at 37°C. The concentration of hemoglobin was determined by alkaline hematin method. The definitions of As and Aw are the same as in the legend to Fig. 1.

The relationship between the strongly immobilized component and hemoglobin content is demonstrated in Fig. 2. The amplitudes, As and Aw, of the strongly immobilized component and the weakly immobilized one were measured from the base line as shown in Fig. 1. The plots of As/Aw values vs. hemoglobin contents showed a hyperbolic curve. Up to the half content of hemoglobin within intact erythrocyte, the values of As/Aw ratio increased with increasing hemoglobin contents. At higher concentrations of hemoglobin, the ratio remained almost constant.

To study the relationship between an appearance of the strongly immobilized component and the changes in membrane proteins, SDS-polyacrylamide gel electrophoresis of membrane proteins was carried out. The electrophoretic patterns of the samples which were characterized by the strongly immobilized component showed the diffuse, nondiscrete bands of high molecular weight due to the cross-linking of membrane proteins

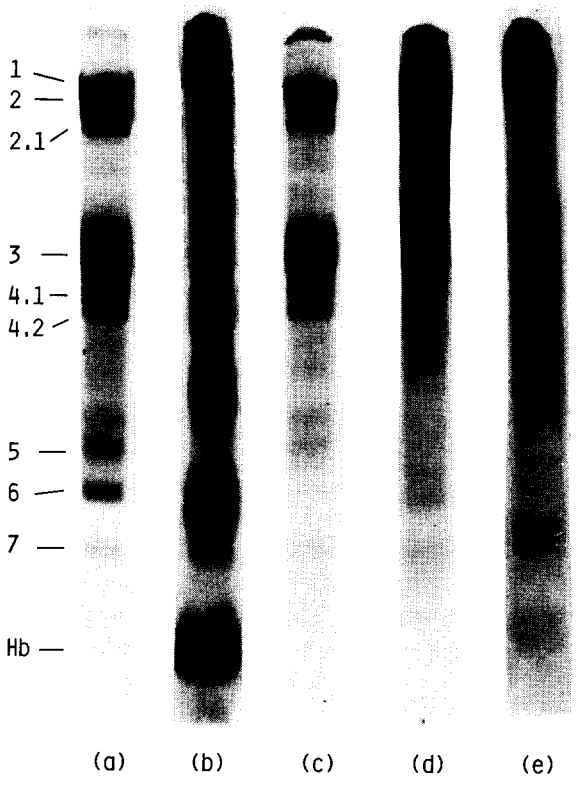

Fig. 3. SDS-polyacrylamide gel electrophoresis of erythrocyte ghosts. Ghosts were prepared by the method of Dodge et al. [13] from intact (a) and 5 mM hydrogen peroxide-treated (b) erythrocytes. Ghosts prepared from intact erythrocytes were treated with 5 mM hydrogen peroxide (c), or 25 mM potassium superoxide (d). Old ghosts (e), the definition is described under Materials and Methods. Hb, hemoglobin.

(Figs. 3b, 3d and 3e). Glutaraldehyde (0.25 M) treatment of ghosts resulted in the cross-linked high molecular weight polymers which could not enter into 5.6% polyacrylamide gel. Hydrogen peroxide (5 mM) treatment of ghosts caused mainly the diminution of band 6 and a high molecular weight polymer on top of the gel (Fig. 3c). In these ghost samples, the weakly immobilized signal was dominantly observed.

In the reaction of erythrocytes with oxidants, the binding of globin to membranes was observed. At higher concentrations of oxidants, lysis occurred. The possibility of the appearance of the strongly immobilized signal due to these phenomena was examined. In the linking of globin to membranes, both phospholipids and membrane proteins may be considered as the linking sites. To study the interaction between hemoglobin and lipids, hemoglobin was entrapped within the liposomes prepared from erythrocytes. Hemoglobin-entrapped liposomes were exposed to hydrogen peroxide (5 mM) so that fresh red color of oxyhemoglobin changed drastically to the characteristic one of methemoglobin, indicating the penetration of hydrogen peroxide into the liposomes. However, the ESR spectrum of the liposomes was identical with that before the oxidation. In 1 mM hydrogen peroxide-treated erythrocytes which were not hemolysed, the ESR spectrum of 12-nitroxide stearic acid alone showed apparently the strongly immobilized signal.

Fig. 4 represents the temperature dependence of the strongly immobilized component obtained for 12-nitroxide stearic acid under various conditions. Their outer hyperfine splitting ($T_{\parallel}$) values in erythrocytes changed about 2 gauss in the temperature range from 5 to 55°C. In 55°C-incubated erythrocytes, the strongly immobilized component was observed obviously, whereas in ghosts no such a signal appeared. The temperature dependence of the $T_{\parallel}$ values in 55°C-treated erythrocytes was

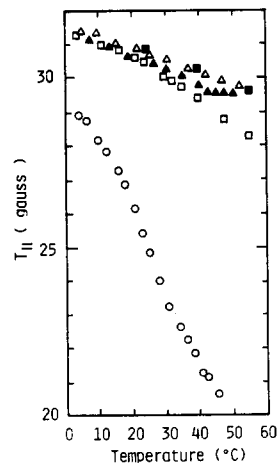

Fig. 4. Temperature dependence of the outer hyperfine splittings ($T_{\parallel}$) of the strongly immobilized component and the weakly immobilized component. Erythrocytes treated with 0.25 M glutaraldehyde (△), 5 mM hydrogen peroxide (▲), or 55°C (■) and 5% bovine serum albumin (□) were spin-labeled with 12-nitroxide stearic acid in phosphate-buffered saline (pH 7.4). In intact erythrocytes, the weakly immobilized component (○) and the strongly immobilized component are observed. However, the $T_{\parallel}$ values of the latter are obscure and are not shown.

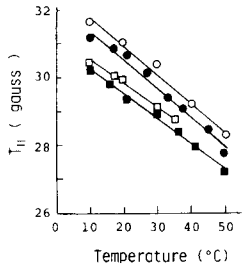

Fig. 5. Temperature dependence of the outer hyperfine splitting (T_{II}) of the strongly immobilized component. Erythrocytes treated with 20 mM H_2O_2 were spin-labeled with 5-nitroxide stearic acid (○) or 16-nitroxide stearic acid (□). The T_{II} value for 16-nitroxide stearic acid could not obtain above 40°C because of the partial overlap of the strongly immobilized component and the weakly immobilized one. Spin labeling of 5% bovine serum albumin was carried out using 5-nitroxide stearic acid (●) or 16-nitroxide stearic acid (■) in phosphate-buffered saline (pH 7.4).

obtained by decreasing the temperature from 55°C, and showed the similar behavior with that in oxidant-treated erythrocytes. The temperature dependence of the T_{II} values of the strongly immobilized component observed in erythrocytes was nearly similar to that in bovine serum albumin up to 40°C. Fig. 5 shows the temperature dependence of the strongly immobilized components for 5-nitroxide stearic acid and 16-nitroxide stearic acid in hydrogen peroxide-treated erythrocytes and bovine serum albumin. The T_{II} values for both labels in the erythrocytes changed about 2 gauss in the temperature range of 10–35°C and their changes were similar to those in bovine serum albumin. In the case of 16-nitroxide stearic acid-labeled erythrocytes, the exact T_{II} value could not obtain above 40°C because of the partial overlap of the strongly immobilized component and the weakly immobilized one, but the former component was observed up to 50°C.

The pH dependence of the T_{II} values of the strongly immobilized component was also examined. The T_{II} values of the signals for 5-nitroxide stearic acid, 12-nitroxide stearic acid and 16-nitroxide stearic acid in both oxidant-treated erythrocytes and bovine serum albumin remained almost constant in the range of pH 5.0 to 9.1 and were 31, 31 and 30 gauss at 20°C, respectively.

Discussion

The ESR spectra of 12-nitroxide stearic acid incorporated into intact erythrocytes show the two components, i.e., the strongly immobilized component and the weakly immobilized one, above 18°C [3]. At higher temperatures, the strongly immobilized component appears predominantly. The peak-to-peak amplitudes of the center field line decrease above 40°C [4]. In the case of ghosts as well as lipid vesicles, however, neither the strongly immobilized component nor the decrease of the amplitude of the line is observed at higher temperatures. Such a difference in temperature dependence between erythrocytes and ghosts was observed in the reactions with oxidants such as glutaraldehyde, hydrogen peroxide, phenylhydrazine and Cu-*o*-phenanthroline. The strongly immobilized signal augmented with increasing the hemoglobin contents within the membranes. These results suggest the necessity of the existence of hemoglobin for the appearance of the strongly immobilized component. The reaction of hemoproteins with peroxides produces hemoprotein radicals [21,22]. By exposing erythrocytes to oxidants, oxyhemoglobin is oxidized to methemoglobin and hemoglobin radical is formed [21,23]. Therefore, free radicals, which contain hemoglobin radical, generated in the reactions of erythrocytes with oxidants may cause the oxidative damage of proteins, i.e., cross-linking of membrane proteins, formation of globin oligomers, etc., as demonstrated by SDS-polyacrylamide gel electrophoresis [24].

By the oxidation of erythrocytes, furthermore, the following phenomena are observed: lysis, lipid peroxidation [24] and linking of hemoglobin to membranes. These attributions on the appearance of the strongly immobilized component were examined as follows. Foremost, the binding of labels to hemoglobin in hemolysate or within erythrocyte may be considered. The ESR spectrum of labeled erythrocytes does not change before and after the hemolysis by the freeze-thaw method, suggesting that the affinity of label for membranes is extremely stronger than that for hemoglobin [4]. In hydrogen peroxide-treated but no hemolysed erythrocytes, 12-nitroxide stearic acid exhibited obviously the strongly immobilized signal but other

spin labels did not. Therefore, the immobilized signal reflects not the direct interaction of the label with hemoglobin due to its penetration into erythrocytes but the state of the membranes. Thus, one possibility due to hemoglobin is excluded. Exposure of erythrocytes to oxidants generates malonyldialdehyde which is the end product in lipid peroxidation. In the vesicles prepared by lipid extraction from erythrocytes which already occurred the lipid peroxidation, no such an immobilized component is observed (Yamaguchi, T. et al., unpublished results). Furthermore, the result of hemoglobin-entrapped liposomes indicates that there is no possibility of appearance of the strongly immobilized component due to the binding of hemoglobin to lipid layer. However, we can not ignore the effects of the linking of hemoglobin to membrane proteins. In the reactions of ghosts with oxidants, potassium superoxide alone, which is generator of superoxide radical, represents the strongly immobilized signal. The electrophoretic pattern of this sample shows the diffuse, nondiscrete bands due to the cross-linking of membrane proteins as demonstrated in oxidant-treated erythrocytes. However, in other oxidative agents, no such an electrophoretic pattern is observed, e.g., in the case of Cu-*o*-phenanthroline, band 3 protein is cross-linked into a dimer by oxidation of sulfhydryl groups to disulfides [25]. These results suggest that the appearance of the strongly immobilized component may be concerned with such cross-linking of membrane proteins as characterized by the diffuse bands.

The temperature dependence of the $T_{\parallel}$ value of the strongly immobilized component observed in oxidized erythrocytes is significantly small as compared with that of the weakly immobilized component [3] and is similar to that of bovine serum albumin. The fact that the strongly immobilized component appeared in the oxidation of erythrocytes has the similar temperature dependence irrespective of the kinds of oxidants used indicates that the membrane damage by these reagents may be occurred through the similar mechanisms.

The pH dependence of the $T_{\parallel}$ value of the strongly immobilized component is different from that of the weakly immobilized one [6]. Namely, the $T_{\parallel}$ values of the strongly immobilized component in oxidized erythrocytes as well as bovine serum albumin remained almost constant in the range of pH 5.0 to 9.1. Therefore, vertical displacement of label molecules in bilayers due to the ionization of their carboxylic group, as indicated in liposomes [26], does not occur because of the lipid-protein interaction.

Such a strongly immobilized component mentioned above appears in a number of natural membranes, i.e., rod outer segment disc membranes [11,12,27], $(Na^+ + K^+)$-ATPase membranes [28], chromatophores [29], etc. The strongly immobilized component observed in these membranes has been attributed to the lipid-protein interactions [9]. A fatty acid spin label contacts with hydrophobic surface of proteins so that the motion of label is significantly restricted. Such a lipid has been proposed as boundary lipid by Jost et al. [10] and distinguished from bulk lipid. The $T_{\parallel}$ values of the strongly immobilized component due to the boundary lipid reveal a considerable temperature dependence [12,27]. Namely, in rod outer segment disk membranes, the $T_{\parallel}$ values change from 29.5 gauss at 0°C to 27.0 gauss at 24°C [12], indicating that the lipids in the first shell are not bound tightly to proteins but are exchangeable with other shell lipids.

Recently, Watts et al. [11,12] have reported on the rigidly immobilized lipid due to the quite different origin with boundary lipid by using 14-nitroxide stearic acid and 16-nitroxide stearoyl maleimide spin labels. Such lipids induced by the extensive bleaching or delipidation of rod outer segment membranes demonstrate very little temperature dependence compared with boundary lipid [12]. They have concluded that such an immobilized signal is attributed to the lipid trapping within aggregated proteins. In order to compare the lipid state of oxidized erythrocytes with that of rod outer segment membrane, similar label molecules should be used. The temperature dependence of the strongly immobilized signal of 16-nitroxide stearic acid incorporated into hydrogen peroxide-treated erythrocytes is similar to that of bovine serum albumin but is significantly different with that of boundary lipids or trapped lipids in rod outer segment membranes. If 16-nitroxide stearic acid exists as a boundary lipid, the strongly immobilized component and the weakly immobilized one collpase above 35°C [30]. However, no

such a collapse was observed in oxidized erythrocytes. These data suggest that the label molecules bound to membrane proteins can not readily exchange with other shell lipids like boundary lipids because of the decrease of lipid fluidity due to cross-linking of membrane proteins. From the temperature dependence of $T_{\parallel}$ values, the lipids in the erythrocytes are not trapped within aggregated proteins as indicated in rod outer segment membranes. Furthermore, the fact that the strongly immobilized component augments with increasing concentration of oxidants indicates that the amount of immobilized lipids is dependent on the degree of cross-linking of proteins. From these results, we propose that the strongly immobilized signal observed in oxidant-treated erythrocytes reflects the interaction of the lipids with the cross-linked products of membrane proteins.

Judging from the data presented up to the present time [3,4,6], 12-nitroxide stearic acid, of these three kinds of fatty acid spin labels, is most sensitive to the changes in membrane proteins. The fact that the strongly immobilized signal observed for 12-nitroxide stearic acid in intact erythrocytes exhibits little temperature dependence [3] suggests that the labels bound to membrane proteins can not readily exchange with other shell lipids, as demonstrated in oxidized erythrocytes.

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