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# SPIN-LABEL STUDIES OF THE OLIGOMERIC STRUCTURE OF BAND 3 PROTEIN IN ERYTHROCYTE MEMBRANES AND IN RECONSTITUTED SYSTEMS

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**A spin-labeled fatty acid (16-doxylstearic acid), linked by an ester bond to a maleimide or a nitrene residue, was covalently attached to band 3 of erythrocyte membranes. The electron spin resonance spectrum of the spin-labeled protein was examined at different temperatures in: (a) whole erythrocyte ghosts; (b) ghosts depleted of spectrin and actin; (c) alkaline-treated ghosts; (d) vesicles made with purified band 3 reassociated with dimyristoylphosphatidylcholine. Most spectra are composite with a major component corresponding to a large overall splitting. The determination of the percentage of the immobilized component was carried out by pairwise subtraction. At low temperatures (1–7°C), the highest fraction of immobilized component was found in dimyristoylphosphatidylcholine vesicles (approx. 100%); alkaline-treated membranes had approx. 75% of the immobilized component at the same temperature; whole erythrocyte, spectrin/actin-depleted and spectrin/actin/ankyrin-depleted ghosts gave identical results (approx. 60% of immobilized component). The immobilized fraction decreased in all samples with increasing temperature or addition of a nonsolubilizing concentration of dodecyl octaethylene glycol monoether. In dimyristoylphosphatidylcholine vesicles, however, the modification in the ratio of the two components was obtained only above the lipid transition temperature (23°C). The strong immobilization of the spin-labeled lipid chain at all temperatures suggested trapping of the lipid chain between proteins. At low temperature, in dimyristoylphosphatidylcholine vesicles or in alkaline-treated ghosts, lipid-protein segregation is likely to take place. In whole erythrocyte ghosts, on the other hand, the large contribution of the motionally restricted component at physiological temperature indicates the oligomeric nature of band 3. Partial dissociation of the oligomers occurs as the temperature is increased, but the presence or absence of cytoskeletal proteins has no influence on the state of oligomerization of band 3.**

## Introduction

Spin-labeled fatty acids, covalently attached to intrinsic membrane proteins through a maleimide, nitrene, or isocyanate linkage, have been used to probe the interaction of integral membrane proteins with the hydrophobic portion of the bilayer. ESR spectra of a nitroxide at the sixteenth position of a fatty acid attached covalently to $Ca^{2+}$-ATPase consisted of two components ('immobilized' and fluid lipid), which in the simplest model are indicative of two states (boundary or trapped lipid and free lipid). In sarcoplasmic reticulum vesicles the immobilized component was interpre-

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; (1,14)-MSL, long chain spin-labeled maleimide (see formula in text); (1,14)-NAP, long chain spin-labeled nitrene (see formula in text); $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether.

ted in terms of lipid trapped between subunits of oligomeric $Ca^{2+}$-ATPase, since very large hyperfine splittings were observed at all temperatures investigated [1–3]. The hydrophobic surface of the membrane protein rhodopsin has been probed with the same maleimide-containing spin-labeled fatty acid, (1,14)-MSL, and the amount of immobilized component was less than found with $Ca^{2+}$-ATPase. For rhodopsin-lipid interactions, interpretations have been advanced postulating a boundary of moderately immobilized hydrocarbon chains surrounding each protein molecule at low temperature ('motionally restricted component') or an entrapment of lipid chains between protein aggregates ('trapped lipid component'), depending on experimental conditions such as bleached versus unbleached state [4–7]. Using (1,14)-MSL Quintanilha et al. [8] found a highly mobile lipid population at the boundary of cytochrome *c* reductase. Finally, Swanson et al. [9] have investigated the hydrophobic boundary of cytochrome *c* oxidase with (1,14)-MSL in reconstituted systems. The interpretation of their data was along the lines of the above discussion, i.e. the spin-label allows the discrimination between aggregated states and non-aggregated states of the proteins.

Band 3, which constitutes about 25% of the total membrane protein of the erythrocyte [10] and forms oligomers in the erythrocyte membrane [11–14] represents a model system for analyzing the effects of protein aggregation on the ESR spectra of covalently attached, hydrophobic spin labels. In the present study two 16-doxylstearate spin labels (Fig. 1) were covalently attached to band 3 via a maleimide or nitrene residue. The effects of protein aggregation on the ESR spectra

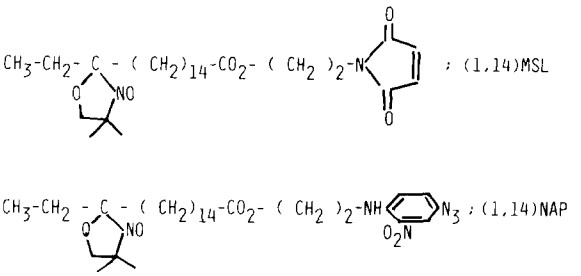


Fig. 1. Structures of the 16-doxylstearate esters used to label band 3, (1,14)-MSL and (1,14)-NAP.

of these probes were examined in reconstituted band 3/DMPC vesicles and in ghost membranes enriched in band 3 by depletion of peripheral proteins. The very large proportion of immobilized lipid component in the ESR spectra at high temperatures is attributed largely to lipid chains trapped in protein oligomers.

## Materials and Methods

*Spin-labels.* The spin-labels (1,14)-MSL and (1,14)-NAP (Fig. 1) were generously provided by Drs. Favre and Fellmann. These molecules were synthesized by the procedures of Favre et al. [4] and Fellmann et al. [1].

*Preparation and spin labeling of ghosts.* Ghosts were prepared from recently outdated blood obtained from a blood bank. Erythrocytes were washed four times with isotonic buffer (5 mM Tris/1 mM EDTA/140 mM NaCl, pH 7.4), and the buffy coat and supernatant were removed by aspiration. Hypotonic lysis of the washed erythrocytes as described by Dodge et al. [15] was performed by rapid mixing in 20 volumes of ice-cold 5 mM sodium phosphate/1 mM EDTA, pH 8.0. The ghosts were washed three times with this buffer (the latter two times in the presence of 20 μg/ml of phenylmethylsulfonyl fluoride (Sigma)), and twice with 5 mM citrate/1 mM EDTA, pH 6.1. Reaction of ghosts with (1,14)-MSL was carried overnight at 4°C by addition of (1,14)-MSL dissolved in absolute ethanol to ghosts suspended in citrate buffer, pH 6.1. The ethanol concentration did not exceed 1.5% by volume. The molar ratio of band 3: (1,14)-MSL was 2:1, assuming that band 3 constitutes 25% of the total ghost protein [10]. Unreacted (1,14)-MSL was removed by washing four times with at least 40 volumes of the following buffers at 0°C: once with 2% fatty acid-poor bovine serum albumin (Sigma) in citrate buffer, pH 6.1, once with 1% fatty acid-poor bovine serum albumin in 5 mM Tris, pH 7.4, then twice with 5 mM Tris buffer, pH 7.4. Spectrin/actin-depleted ghosts were prepared from the labeled ghosts as described by Elgsaeter et al. [16]; the ionic strength was decreased by washing once at 0°C in 40 volumes of 0.3 mM sodium phosphate, pH 7.6, and then suspending the ghosts in 0.3 mM sodium phosphate, pH 7.6, followed by shaking at

37°C for 30 min. To determine the concentration of spin label incorporated into the crude cytoskeleton protein, the supernatant obtained after the membranes were pelleted was concentrated by ultrafiltration (Minicon-B15 concentration, Amincon). The procedure of Tyler and Branton [17] was used to remove band 6 from spectrin/actin-depleted ghosts (either prior to or after reaction with (1,14)-MSL). Labeled ghosts were depleted of bands 2.1 and 4.1 by the procedure of Wolosin et al. [18]. Residual membranes from ghosts depleted of bands 1, 2, 2.1, 4.1, 5, and 6 were collected by centrifugation for 20 min at 45000 rpm in a Beckman Ti 50 rotor. Alkaline-treated ghosts were prepared as described by Sakaki et al. [19]. Band 3 was assumed to constitute 75% by weight of the total protein in ghosts depleted of peripheral proteins. To prepare spin-labeled band 3/DMPC (Sigma) reconstituted vesicles, alkaline-treated ghosts were treated with (1,14)-MSL using a spin label: band 3 ratio of 3:1. The reaction with (1,14)-MSL was carried out in 5 mM imidazole/1 mM EDTA, pH 6.0, for 1.5 h at 37°C. The membranes were collected by centrifugation, washed once with 5 mM imidazole/1 mM EDTA, pH 6.0, containing 2% fatty-acid-poor bovine serum albumin and then twice with 5 mM imidazole/1 mM EDTA, pH 7.4. The reaction with (1,14)-NAP was carried out using an aqueous dispersion of the spin label prepared by sonicating in the dark an ethanolic solution of (1,14)-NAP (2 mM final concentration) in 5 mM imidazole/1 mM EDTA, pH 7.4. This aqueous dispersion was mixed with alkaline-treated ghosts suspended in the same buffer at a band 3: (1,14)-NAP molar ratio of 2:1. The suspension was irradiated at room temperature with an argon laser (Spectra Physics model 164) for three 20-s periods. Spin-labeled band 3 was isolated, purified, and reconstituted with DMPC (Sigma) vesicles as described elsewhere [19].

*Analytical procedures.* Protein was assayed according to the method of Lowry et al. [20] using bovine serum albumin as standard. For measurement of the amount of (1,14)-MSL associated with the lipids, extraction of lipids from a sample of labeled ghost membranes was performed according to Bligh and Dyer [21] and Rose and Oklander [22]. Phospholipid phosphorus was measured by the method of Rouser et al. [23] and cholesterol was determined by the method of Zlatkis and Zak [24]. SDS-polyacrylamide gel electrophoresis was conducted according to Laemmli [25]. After electrophoresis, gels were stained with Coomassie blue, which confirmed that the expected protein bands were substantially released by the various extraction procedures used.

*ESR spectroscopy.* ESR spectra were recorded on a Varian E-109 spectrometer fitted with a variable temperature accessory. The temperature was measured with a copper-constantan thermocouple inserted at the top of the 50-$\mu$l quartz cell just above the microwave cavity. Double integrations of the experimental spectra were performed using a Tektronix 4051 computer connected to the spectrometer.

## Results

*Binding of (1,14)-MSL to ghosts*

The reaction of (1,14)-MSL with ghosts was conducted at pH 6.1 to minimize the nonspecific reaction with amino groups. The sulfhydryl specificity of the maleimide spin label was also increased by using low label concentrations and low temperature. When labeling was done using a molar ratio of (1,14)-MSL: band 3 of 1.6, double integration of the ESR signal of the lipids extracted from the labeled ghosts revealed that membrane lipids contributed a maximum of 10% to the total signal of the ghosts. (When labeling was performed at a (1,14)-MSL: band 3 molar ratio of 0.5, the lipids were unlabeled, but the total extent of labeling was only 25% of that obtained at a ratio of 1.6.) The cytoskeleton proteins contributed a maximum of 10% of the total ghosts signal, as determined by double integration of the ESR signal in concentrated spectrin/actin extracts recovered in the supernatant of the low ionic strength extraction. The double integral did not change on removal of band 6, indicating that binding to the reactive sulfhydryl group of glyceraldehyde-3-phosphate dehydrogenase did not occur. Furthermore, the double integral of the signal per mg of protein was not affected by trypsin treatment of labeled, spectrin-actin-depleted ghosts, indicating that the hydrophobic spin label did not bind to an extramembranous fragment. Moreover, the ESR spectra of whole ghosts, spectrin/actin-depleted

ghosts, and ghosts labeled subsequent to extraction of bands 1, 2, 5 and 6 were virtually identical at all temperatures we examined. Since glycophorin does not contain a sulfhydryl group, these results suggest a preferential reaction of (1,14)-MSL with band 3 in ghosts. Double integration of the ghost signal indicated that the extent of (1,14)-MSL incorporation into ghosts was approx. 0.3–0.7 mol per mol of band 3 protein, assuming that binding to other membrane components was negligible.

*ESR lineshapes*

The ESR spectra of (1,14)-MSL in erythrocyte ghosts depleted of spectrin and actin, and in band 3/DMPC vesicles are shown in Figs. 2 and 3. All of the spectra have the appearance of a superposition of two components. One component corresponds to strongly immobilized probes, while the other component corresponds to probes experiencing a high degree of mobility. For each temperature the ratio of the two components is different in erythrocyte ghosts depleted of spectrin and actin and in band 3/DMPC vesicles. Also, in a given sample this ratio appears to vary with the temperature. The spectrum of (1,14)-MSL in ghosts was not altered by addition of the water-soluble broadening agent $Ni^{2+}$, suggesting immersion of the spin label in the hydrophobic phase of the membrane.

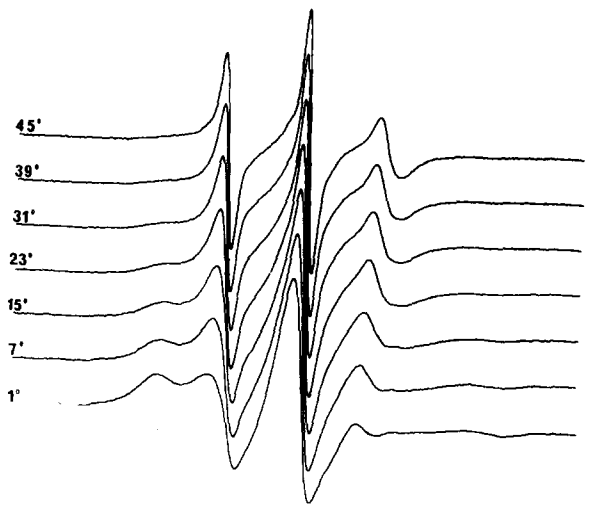


Fig. 2. ESR spectra of (1,14)-MSL in spectrin/actin-depleted ghosts as function of temperature.

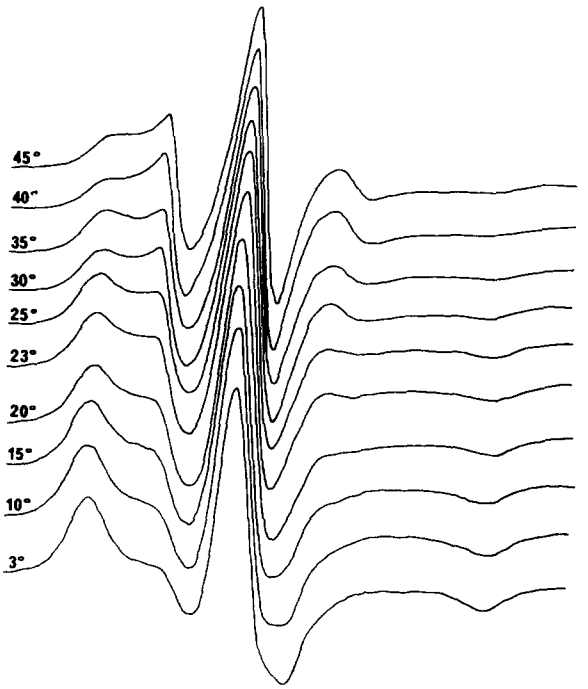


Fig. 3. ESR spectra of (1,14)-MSL in band 3/DMPC recombinant vesicles as a function of temperature. The protein to lipid ratio was 1:270.

Similar spectra have been recorded with whole erythrocyte ghosts, trypsin-treated ghosts and ghosts selectively depleted of ankyrin and band 4.1 by sequential salt extraction. The latter membrane systems gave spectra virtually identical at each temperature to the spectra obtained in spectrin/actin-depleted ghosts. On the other hand, the ESR spectra obtained with alkaline-treated ghosts or with spectrin/actin-depleted ghosts in the presence of $Ca^{2+}$ and ionophore A 23187 have a significantly larger fraction of the immobilized component. For example, the spectra at 23°C of spectrin/actin-depleted and alkaline-treated ghosts are compared in Fig. 4 a and b. By appropriate combination of spectra a and b by computer, the narrow component is eliminated and a 'pure immobilized component' (spectrum c, Fig. 4) is generated. Spectrum d was obtained by combination of the spectra of spectrin/actin-depleted ghosts with and without $Ca^{2+}$ and ionophore A23187.

As already observed in sarcoplasmic reticulum

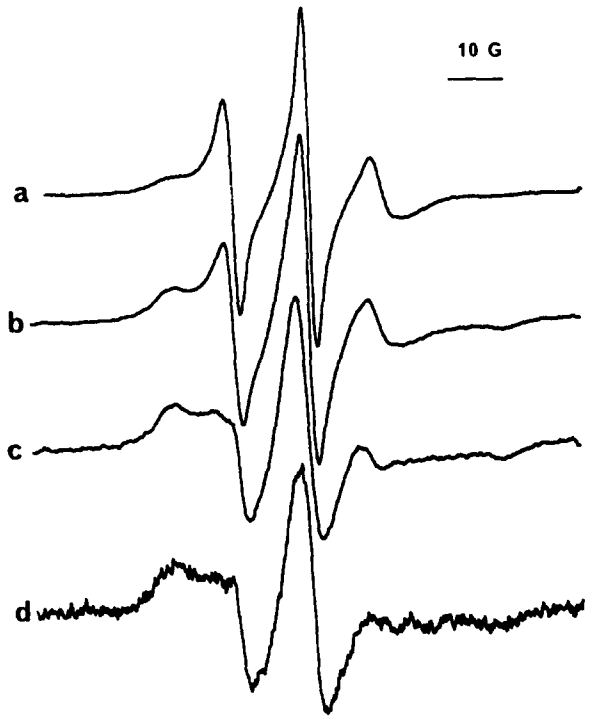


Fig. 4. Comparison of the ESR spectra obtained in ghosts at 23°C under different conditions of protein aggregation. (a) spectrin/actin-depleted ghosts; (b) alkaline-treated ghosts; (c) pairwise combination of spectrum (a) and spectrum (b); (d) pairwise combination of spectrum (a) with the spectrum obtained in spectrin/actin-depleted ghosts after addition of 2 mM $Ca^{2+}$ and A23187 (5 $\mu$g/ml) (spectrum not shown).

vesicles [2], the addition of small amounts of the non-ionic detergent $C_{12}E_8$ to membranes labeled with (1,14)-MSL results in an apparent increase of the fraction of mobile component (Fig. 5).

In some of the above systems we have used also (1,14)-NAP to label covalently the proteins. The ESR spectrum of (1,14)-NAP in alkaline-trated ghosts is shown in Fig. 6 before and after illumination. The sample was washed with fatty acid-free bovine serum albumin after illumination to remove unreacted labels. Band 3 protein labeled with (1,14)-NAP has also been purified and reconstituted in DMPC. All spectra obtained with the nitrene derivative are virtually identical to those obtained with the maleimide derivative.

*Estimation of the fraction of immobilized component*

In order to evaluate the ratio of the two ESR components in the various systems, and its dependence with temperature, it is necessary to determine the lineshape of each component at each temperature. The immobilized component is clearly

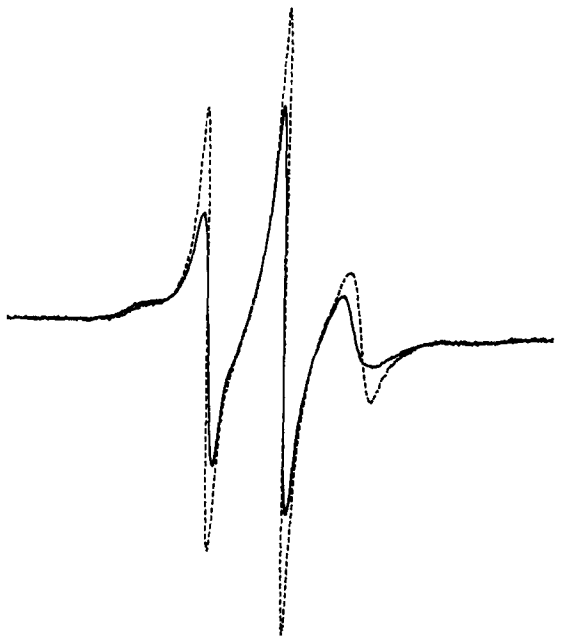

Fig. 5. Influence of the addition of a nonsolubilizing concentration of the nonionic detergent $C_{12}E_8$ on the ESR spectrum of (1,14)-MSL in spectrin/actin-depleted ghosts. The solid curve corresponds to the spectrum at 23°C before addition of detergent. The dotted curve is the spectrum at 23°C after addition of 0.6 mg $C_{12}E_8$ per mg protein.

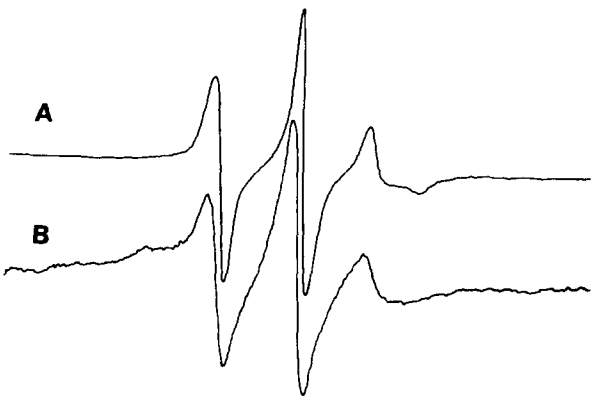


Fig. 6. ESR spectra of (1,14)-NAP in alkaline-treated ghosts at 23°C. Spectrum A, before illumination; spectrum B, after illumination at 486 nm at 0°C, followed by washing with fatty acid-free bovine serum albumin.

visible at low temperature. In particular, the overall splitting can be measured unambiguously. Thus at low temperature, it would be possible to use a broad spectrum determined from computer simulation or from a set of reference spectra. However, for most spectra recorded at or above 23°C, with the exception of spectra recorded with DMPC/band 3 recombinants, the overall splitting is difficult to measure and the proper lineshape of the immobilized component cannot be determined unambiguously. Consequently, we have used a pairwise subtraction method in which the spectra are recorded under different conditions of protein aggregation at each temperature, i.e., with a variable ratio of mobile and immobile components. Then a linear combination of the spectra is carried out, allowing us to generate a 'pure immobilized component' and a 'pure mobile component'. This procedure, although somewhat arbitrary in the choice of the exact final lineshape, leads to a reliable quantitation. Indeed the inaccuracy in lineshape is responsible for an uncertainty in the percentage of immobilized component not larger than 5%. As indicated already, increased aggregation was achieved using high pH (alkaline-treated ghosts) or with A23187-mediated $Ca^{2+}$ incorporation into the ghosts [26]. The immobilized components obtained at 23°C by both of these approaches are shown in Fig. 4. The two spectra are reasonably similar. Other spectra have been obtained from −1 to 39°C. The overall splittings of the immobilized components are given in Table I.

A third procedure for the determination of the immobilized components was employed at low temperatures with spectrin/actin-depleted ghost and at all tempearutres with DPMC/band 3 vesicles. This procedure, which has been outlined previously [2], consists of combining spectra from

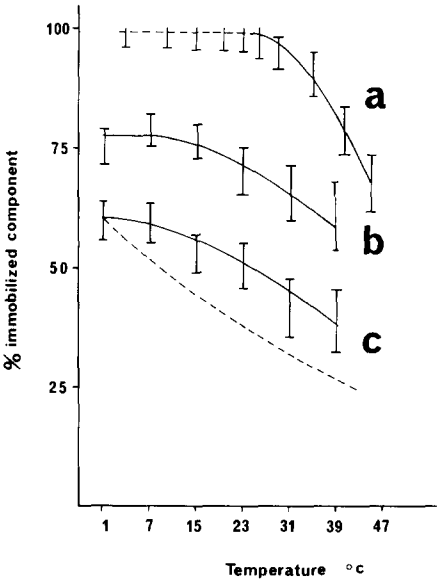


Fig. 7. Temperature dependence of the percentage of immobilized component of (1,14)-MSL in band 3/DMPC recombinants (a), alkaline-treated ghosts (b), and spectrin/actin-depleted ghosts (c). The percentage was obtained by subtraction of an immobilized component determined for each temperature. The dotted curve for spectrin/actin-depleted ghosts is the result of a quantitation performed with fixed 'immobilized component' (determined at −1°C).

the same sample that were recorded at two slightly different temperatures. As long as the lineshapes of the immobilized and mobile components are only moderately temperature dependent, an 'average immobilized component' and an 'average mobile component' can be determined in each range of temperature, and the percentage of the two components can be determined. This procedure is inapplicable at high temperature if the mobile component is the dominant one because its lineshape is too sensitive to temperature; the ampli-

TABLE I

TEMPERATURE DEPENDENCE OF THE OUTER HYPERFINE SPLITTING OF THE IMMOBILIZED COMPONENT IN NATIVE ERYTHROCYTE GHOSTS AND SPECTRIN/ACTIN-DEPLETED GHOSTS

The immobilized component is determined at each temperature by linear combination of the spectra obtained with (1,14)-MSL in spectrin/actin-depleted ghosts and in alkaline-treated ghost.

| Temperature (°C) | −1 | 7 | 15 | 23 | 31 | 39 |
|---|---|---|---|---|---|---|
| $2T'_{\parallel}$ (Gauss) | 62.0±0.3 | 61.5±0.5 | 60.0±0.5 | 58.8±0.8 | 57.2±1.0 | 56.6±1.5 |

tude of the immobilized component generated by subtraction then becomes comparable to the amplitude of the spectral 'noise' produced by subtraction of the two mobile components. In practice, in whole erythrocyte ghosts and spectrin/actin-depleted ghosts, we found that 15°C was the highest temperature at which this procedure could be used. Similarly, we found that the addition of $C_{12}E_8$ could bearly be used to determine the lineshape of the immobilized component because of the modification of the mobile component lineshape resulting from the addition of detergent (see Fig. 5).

Fig. 7 shows the results of the quantitation of the fraction of immobilized component in the various systems investigated. This figure contains the data obtained using the different procedures outlined above. In the spectrin/actin-depleted samples we have carried out an additional estimation of the fraction of immobilized component (results indicated by the dotted curve). This estimation uses the same immobilized component for all temperatures. The immobilized component at −1°C was employed for this calculation. This technique gives an underestimate of the percentage of immobilized component as pointed out by Brotherus et al. [27]. Each procedure shows a temperature-controlled variation of the fraction of immobilized component.

## Discussion

*Specificity of the labeling*

When erythrocyte membranes are reacted with *N*-ethylmaleimide several proteins are labeled, the major ones being spectrin and band 3 [28]. Hydrophilic maleimide spin labels have been shown to bind to extramembranous sites [29,30]. However, the presence of the hydrophobic chain in (1,14)-MSL is very likely to position the reacting center and prevent MSL penetration into the interior of the ghost. Indeed, (1,14)-MSL has little affinity toward the extrinsic protein spectrin, as judged by ionic extraction of spectrin. Since band 3 is the only major intrinsic protein possessing a sulfhydryl group, we conclude that the latter protein is labeled predominantly by (1,14)-MSL, even in intact erythrocyte ghosts. This is confirmed by the identity of the spectral lineshapes and spin-labeling ratios in erythrocyte ghosts and erythrocyte ghosts depleted of spectrin and actin, as we have seen above, and by the close values of the ratios of spin label to band 3 before and after purification of the protein by removal of extrinsic proteins. It is noteworthy that covalent binding to phosphatidylethanolamine could not be totally avoided. Spin-labeled lipid was estimated to represent at most 10% of the spectra after washing with fatty acid-free albumin. Removal of the 42 kDa cytoplasmic domain of band 3 by trypsin treatment does not modify the binding ratio of (1,14)-MSL to the membrane. The spin label thus binds to the intrinsic part of band 3. According to Ramjeesingh et al. [31], the later contains three sulfhydryl groups, two in the 35 kDa segment. All three sulfhydryl groups may in principle constitute binding sites for (1,14)-MSL. The third sulfhydryl group has been shown to be non reactive to *N*-ethylmaleimide [32,33] but this may not hold for (1,14)-MSL. Previous work on rhodopsin [4] has shown that (1,14)-MSL may bind to sites not accessible to *N*-ethylmaleimide.

Nitrene derivatives can in principle react with proteins devoid of free sulfhydryl groups. Therefore, in alkaline-treated ghosts (1,14)-NAP might be susceptible to reaction with glycophorin as well as with band 3. However, nitrenes have some affinity for sulfhydryl groups [34] and, since the lifetime of the excited molecule (approx. $10^{-4}$ s) allows the molecule time to diffuse until it reaches a sulfhydryl group, (1,14)-NAP is likely to bind to the same sites as the maleimide derivative with the same fatty acid chain. This would explain why all of the qualitative and quantitative results obtained with illuminated (1,14)-NAP and with (1,4)-MSL are so similar. The same conclusion was reached after a comparative study of the two spin labels in sarcoplasmic reticulum vesicles [1,2].

*Significance of the immobilized component*

The ESR spectra obtained with (1,14)-MSL or (1,14)-NAP, covalently attached to band 3 protein, are characterized by two components in erythrocyte membranes and in reconstituted systems. The major component in almost all instances is the immobilized one. This result is comparable to what was found with $Ca^{2+}$-ATPase [1,2,3], bleached rhodopsin [4] and aggregated cytochrome oxidase [9]. It is markedly different from what was ob-

tained with (1,14)-MSL attached to rhodopsin in intact discs [4] or in reconstituted systems above the phospholipid phase transition temperature [5,6]. It is also different from the results obtained by Swanson et al. [9] in reconstituted systems containing cytochrome oxidase in nonaggregated form. In the latter systems the spectrum is composed essentially, if not uniquely, of narrow lines.

The interpretation of a strong immobilization of a lipid chain in the direct vicinity of an intrinsic protein has been discussed elsewhere [1–9]. It was proposed that the very broad component visible at all temperatures in some of the systems mentioned above corresponded to trapped lipid chains within protein aggregates or protein oligomers (Fig. 8). Here again we suggest that lipid trapping is mostly responsible for the large immobilized component at the boundary of band 3. However, the temperature dependence of the outermost splitting in erythrocyte ghosts (see Table I) indicates that the immobilization of the probe attached to band 3 is less complete than when it is attached to $Ca^{2+}$-ATPase [2]. It is quite reasonable to assume that different oligomers have different configurations; thus a lipid intercalated between the subunits of band 3 oligomer may experience more motion than a lipid intercalated between $Ca^{2+}$-ATPase subunits. We consider the following arguments to favor chain trapping between subunits of band 3 or between oligomeric complexes: (i) Alkaline-treated ghosts are known to contain partially aggregated band 3 protein [37]. Such treatment increased the fraction of motionally restricted component as seen with (1,14)-MSL without significantly increasing the outermost splitting.

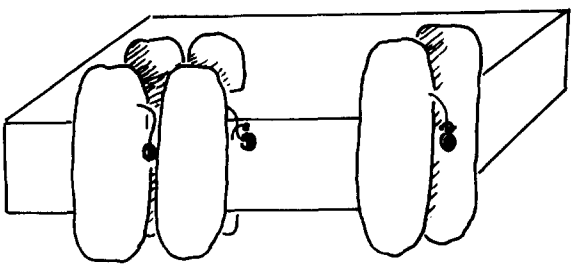

Fig. 8. Schematic representation of influence of the state of oligomerization of band 3 on the mobility of (1,14)-MSL. In the tetrameric state (left), (e.g. dimer of dimers) a fraction of the probes is immobilized because of trapping. The dissociation to a smaller oligomer (right) results in an increase of the mobile fraction. On the other hand, aggregation of the proteins in domains of low lipid content, which may take place at low temperature, would increase the fraction of immobilized probes.

(ii) The motionally restricted component which can be seen with the same probe at the boundary of a monomeric protein such as rhodopsin corresponds to only 40% of the signal at $-1°C$ and is bearly detectable at 39°C [5]. Quantitative results obtained with a probe closer to the ester group are not directly comparable since obviously the extent of immobilization should increase when the probe is moved toward the binding site.

In conclusion we believe that protein-protein interactions are at least partially responsible for the large percentage of immobilized component at the boundary of band 3.

The protein-protein interactions observed in DMPC/band 3 vesicles probably correspond to a temperature-induced aggregation of proteins. Segregation of membrane proteins below the phospholipid phase transition temperature is a well-established phenomenon, having been observed in reconstituted systems containing, for example, rhodopsin [35] or $Ca^{2+}$-ATPase [36]. In alkaline-treated ghosts aggregation in intramembrane particles is strongly temperature dependent [37]. Thus the large hyperfine splittings and high fraction of immobilized component observed in alkaline-treated ghosts and band 3/DMPC recombinant vesicles are consistent with clustering of proteins induced by their insolubility in the gel phase. However, a large percentage of immobilized component persists at 39°C (Fig. 7), suggesting a large degree of protein-protein contacts in the physiological temperature range. In addition, protein aggregation at low temperature has not been reported in native erythrocyte ghosts or in spectrin/actin-depleted ghosts. The large fraction of protein-protein contact that persists under physiological conditions is therefore interpreted in terms of protein oligomers rather than phases containing domains of aggregated proteins coexisting with protein-free lipid areas. The same labels were found to provide evidence for oligomeric complexes of $Ca^{2+}$-ATPase in sarcoplasmic reticulum membranes [1,2].

Several authors have suggested the oligomerization of band 3 to dimers and, to some extent, tetramers [11–14]. There are also reports of the

association of band 3 with glycophorin [38,39] and $(Na^+ + K^+)$-ATPase [40]. The immobilization of the spin-labeled fatty acid at the boundary of band 3 protein could be attributed to trapping of lipid between band 3 and some other intrinsic proteins. The results shown with purified band 3 (Fig. 3) suggest that band 3 oligomeric complexes in ghosts are principally responsible fort he trapping of (1,14)-MSL.

An alternative explanation for the strong immobilization of (1,14)-MSL bound to band 3 is trapping within the hydrophobic part of the band 3 monomer. Band 3 is known to possess several membrane spanning segments [31] and it is conceivable that intercalation of the labeled chain between the latter could promote immobilization. Such a process may indeed account for a fraction of the immobilized component. However, the increase of the fraction of this component observed after alkaline treatment of ghosts or in DMPC/band 3 recombinants is more likely explained by protein-protein associations. Furthermore it should be noted that neither with monomeric rhodopsin [4,5] nor with non aggregated cytochrome oxidase [9], which both possess several membrane spanning domains, is an immobilized component observed at physiological temperature. Only in this cases were protein-protein associations occur [1,2,6,9] has this component been observed.

*Influence of cytoskeletal proteins on band 3 oligomerization*

It is quite striking that low ionic strength extraction of spectrin and actin from ghosts did not affect the ESR spectrum of (1,14)-MSL compared with the spectrum in whole erythrocyte ghosts. Thus the effect of alkaline treatment on band 3 aggregation does not result solely from the release of spectrin/actin that accompanies alkaline treatment. Depletion of ankyrin and band 4.1 also had no effect on the spectra of (1,14)-MSL at all temperatures studied. In accordance with our results, Sakaki et al. [19] recently found that these proteins did not affect the spectrum of (1,14)-MSL in band 3/DMPC vesicles.

Our results with (1,14)-MSL may seem, however, to contrast with published results concerning the influence of cytoskeletal proteins on band 3 rotational and lateral diffusion. Nigg and Cherry [41] found that (at least) bands 2.1, 4.1 and 4.2 influenced band 3 rotation. Similarly, Golan and Veatch [42] and Fowler and Branton [43] suggested that reversible binding of spectrin (via band 2.1) with band 3 may control the lateral diffusion of a fraction of band 3; the evidence supporting the modulation of band 3 lateral movement by cytoskeletal proteins has been reviewed recently [44]. But it should be noted that our results give information only about the extent of stable association between intrinsic proteins, i.e., the extent of oligomerization. Our results show that the cytoskeletal proteins do not control the state of oligomerization of band 3.

*Influence of temperature and nonsolubilizing concentrations of detergent*

Fig. 7 shows that in all types of preparations the fraction of immobilized component decreases when temperature is increased. This cannot be attributed to an artifact associated with a temperature-dependent change of lineshape of the immobilized component, since we determined the lineshape at each temperature. The variation of the percentage of immobilized component in DMPC/band 3 recombinants or in alkaline-treated membranes is consistent with the known temperature-controlled aggregation of band 3 in such systems [37]. One is tempted to conclude that the decrease in the percentage of immobilized component seen with whole erythrocyte ghosts or with spectrin/actin-depleted membranes arises because of the dissociation of band 3 oligomers [45]. This conclusion, however, should be considered cautiously, at least as far as any quantitation is attempted.

Davoust and Devaux [46] have shown recently that the spectrum of (1,14)-MSL covalently attached at the boundary of a monomeric protein (rhodopsin) is composed of two components: a lipid bilayer component with narrow lines and a 'motionally reduced component', which is much broader. At high temperature, these two components exchange rapidly on the time scale of ESR; this produces a decrease in the splitting of the broader component. However, spectral simulations show that the increase of the exchange rate is not sufficient to account for the spectral modifications unless the ratio of the two component is also modified when the temperature is increased. This

finding can be explained if one assumes that the methyl terminal region of the linked fatty acid explores a larger domain of the bilayer at high temperature than at low temperature. Thus both the exchange rate and the proportions of the two states vary with temperature. As a result, when the temperature is increased, a decreasing contribution of the motionally reduced component is expected even for (1,14)-MSL bound to band 3. We conclude that even if the state of oligomerization of band 3 were constant with temperature, a change in the ratio of mobile vs. immobile components would still be observed. Since it is very difficult to predict the amplitude of such effects, one is forced to conclude that the dissociation of band 3 oligomers with temperature is compatible with our results, yet it is not unambiguously proven.

The modification of viscosity at a fixed temperature produced by the addition of $C_{12}E_8$ may also affect the relative proportion of the mobile component of (1,14)-MSL. Thus the change in ratio of the two components is perhaps not associated only with a change of oligomerization following the addition of detergent. However, it should be noted that a very similar nonionic detergent ($C_{12}E_9$) was shown recently to dissociate bovine band 3 in the solubilized form [47].

## Conclusions

The covalent binding of (1,14)-MSL to band 3 in erythrocyte ghosts or in reconstituted systems allowed us to to demonstrate the oligomeric nature of this protein in its membranous state. The temperature study is consistent with partial dissociation of the oligomer at high temperatures. These results confirm the results of Nigg and Cherry [42] obtained by a different dynamical method. In addition, our results show that the dissociation of oligomers is not influenced by the cytoskeletal proteins.

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