Spin Label Study of Apomembranes and Purple Membranes

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Three spin-labelled fatty acids were used to detect the dynamics of lipid bilayer of apomembranes and purple membranes. It was found that ESR spectra of spin labels bound to apomembranes showed a temperature-induced changes rather similar to those seen with purple membranes. At the same time, however, the values of hyperfine splitting parameter $2T_m$ were lower as compared to purple membranes. The results pointed out that the removal of the retinal from purple membranes affects the dynamics of lipid bilayer and apomembranes were more rigid structure than those of purple membranes.

Introduction

The cellular membranes of *Halobacterium halobium,* known as purple membranes consist of a single protein bacteriorhodopsin tightly-packed in a two-dimensional hexagonal crystalline lattice of trimers, surrounded by only 30 lipid molecules [1]. After treatment of PM with hydroxylamine under illumination the colorless chromophore-free membranes, named apomembranes could be obtained [2]. The process of bleaching is reversible. Upon addition of retinal, BR regenerates spontaneously, accompanied with the restoration of the characteristic absorption band and the two-dimensional hexagonal array [3, 4]. It has been shown however, that some structural differences exist between membranes containing BR and BO [5]. The apoprotein in bleached membranes does not possess hexagonal lattice [6]. The removal of the retinal caused changes in the tilting of the helical axis of the polypeptide chain [7], in the fluorescence quantum yield [8] and in the charge asymmetry of membranes [9]. At the same time, although BR undergoes appreciable tertiary structural changes upon bleaching, no significant changes in secondary structure has been detected [10]. Recently, it has been shown, that the native structure of BR is maintained largely as a result of interactions between the retinal and the apoprotein [11]. It was concluded that the retinal serves as the most important factor in stabilizing the BR molecules and regulates the tertiary rather than secondary structure of BR. However, the important role of surrounding lipids in this process could not be also excluded [12]. Study of structural feature of apomembranes is a very essential for the reconstitution of artificial BR [13] and for understanding of the role of chromophore-protein interaction in functioning of BR [14].

The purpose of the study presented is to elucidate the problem to what extent the removal of retinal affects the dynamics and structure of lipid bilayer. We investigate the temperature-induced changes of the fluidity of both PM and apomembranes using three spin probes labelled at different depth of fatty acid chain.

Materials and Methods

Fatty acid was labelled according to Rozanzev [15] and spin probe 2,2,6,6-tetramethyl-4-oxysteronoylpiperid-1-oxyl (SSL) was obtained. The fatty acid spin probe 5-doxyl stearate (5-DS) and 16-doxyl stearate (16-DS) were purchased from Sigma. Purple membranes were isolated from *Halobacterium halobium* according to Oesterhelt *et al.* [16]. Apomembranes were prepared from purple membranes following the procedure in ref. [17]. After bleaching (controlled spectrophotometrically), the samples were centrifuged and washed with water several times to eliminate residual acid. Purple and apomembrane suspensions (10 mg/ml) were incubated with spin probes (10^{-4} M) for 5 h and centrifuged. The obtained samples were suspended in 6.6 mM phosphate buffer, pH 7.6. The

Abbreviations: PM, purple membranes; BR, bacteriorhodopsin; BO, bacterioopsin; ESR, electron spin resonance.

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ESR spectra were recorded on Bruker spectrometer equipped with N2 gas flow temperature controlled system. The spectrometer operating parameters were: frequency 9.5 GHz, modulation frequency 100 kHz, modulation amplitude 1 G.

Results and Discussion

The spectra of 5-DS spin label bound to purple and apomembranes are presented in Fig. 1. The movement of the spin label was markedly sensitive to the temperature in both membrane samples. ESR spectra at low temperature were characterized by a large amount of a tightly immobilized component. As the temperature raises the intensity of the low field peak of ESR spectra gradually decreases. The membrane structural ordering can be precisely analyzed using the order parameter S [18]. This parameter can be determined from the experimental spectra. Unfortunately, at elevated temperatures the distance between the low field minimum and the high field maximum ($2T_{\perp}$) could not be always determined correctly. Thus, we evaluated the hyperfine splitting parameter $2T_m$ as an indicator for the motion of spin label and as a measure of the fluidity of lipid bilayer [19]. The temperature dependence of $2T_m$ for purple and apomembranes are presented in Fig. 2. The mobility of the spin probe increases with the raise of temperature in both membrane samples. The values of $2T_m$ for 5-DS bound to PM were slightly lower than those for apomembranes over all temperature range studied. These results suggest a more rigid environment of 5-DS in apomembranes in comparison with PM. While the temperature dependence of $2T_m$ for 5-DS bound to PM shows a discontinuity at 23 °C, it was not evident in the case of apomembranes. The values of $2T_m$ for 5-DS bound to PM are in good agreement with data previously reported [20]. ESR spectra of 16-DS bound to apomembranes are shown on Fig. 3. At temperatures above 10 °C broadening of the low field peak occurs, suggesting that 16-DS spin label is distributed into more than one environment. The appearance of non-base isoclinic points in ESR spectra of 16-DS bound to apomembranes suggest also that the recorded spectra are superposition of two components: tightly and weakly immobilized. The former component reduces with the raises of the temperatures. No significant difference in the shape of ESR of 16-DS bound to PM as compared to apomembranes was found (data not shown). The experimental data for PM are in agreement with the results reported in ref. [21]. The authors have related the tightly immobilized component to boundary lipids, which

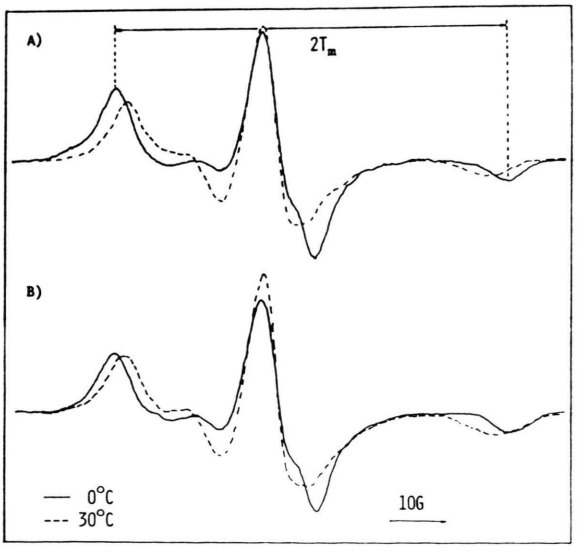

Fig. 1. ESR spectra of 5-doxyl stearate bound to purple membranes (A) and apomembranes (B). Concentration of membrane samples 10 mg/ml.

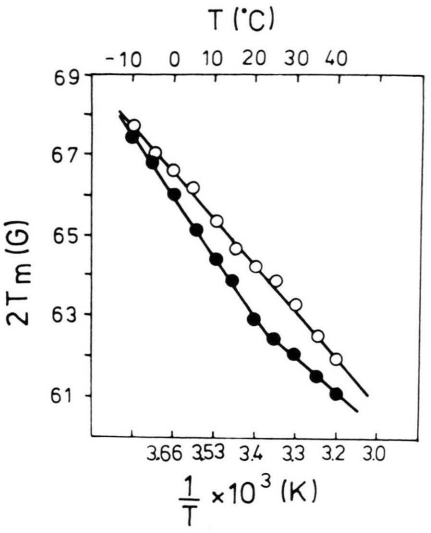

Fig. 2. The effect of temperature on the hyperfine splitting parameter $2T_m$ of 5-doxyl stearate bound to purple membranes (●) and apomembranes (○).

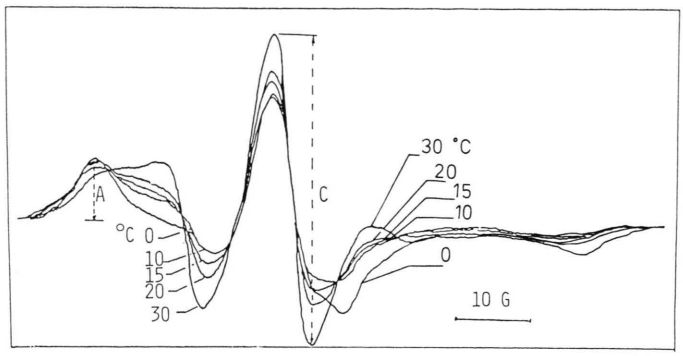

Fig. 3. ESR spectra of 16-doxyl stearate bound to apomembranes (10 mg/ml) at different temperatures.

are closely associated with the protein. As mentioned above a broadening of the low field peak of ESR spectra for both membrane samples was observed. Since at higher temperatures the broadening was too great to allow a resolution of $2T_m$, an evidence only for some values was given. Comparison of the data for the hyperfine splitting parameter $2T_m$ of 16-DS bound to apomembranes show much larger values (65.5 G at 0 °C; 64.42 G at 10 °C), than those for PM (63.68 G at 0 °C; 60.39 G at 10 °C), respectively. As an additional semiquantitative measure of lipid fluidity we used the ratio of the low field to the central field line height amplitudes (A/C) [22]. The values of A/C decreases as motional freedom of the label increases. The data for A/C were 0.27 and 0.29 at 5 °C and 0.17 and 0.22 at 22 °C, for PM and apomembranes, respectively. The values of both spectral parameters ($2T_m$ and A/C) reveal more rigid environment of 16-DS spin label bound to apomembranes as compared to PM.

While 5-DS and 16-DS allow to probe the mobility of the lipid acyl chain deeply in the bilayer, the SSL spin label is located in the head group region of the lipid bilayer and thus is exposed to a polar aqueous environment. ESR spectra of SSL label bound to PM exhibit two components: strongly immobilized and weakly immobilized (Fig. 4). The values of both depend strongly on temperature and the strongly immobilized component diminishes above 15 °C. The values of the maximal hyperfine splitting $2T_m$ at 0 °C were 66 G for SSL, 66.2 G for 5-DS and 63.68 G for 16-DS, respectively. Consistent with the results reported previously [20] these experiments point out a very rigid structure of PM deeply in the lipid bilayer. The removal of retinal does not change essentially the shape of ESR spectra compared to those recorded with spin labels bound to PM. ESR spectra of both purple and apomembranes were characterized by common spectral feature: i) the presence of two components; ii) the decrease of tightly immobilized component with the raise of the temperature; iii) the lack of evident high field maximum in ESR spectra of 5-DS, which makes it impossible to present data for $2T_\perp$ parameter; iv) the broadening of the low field maximum at higher temperatures in ESR spectra of 16-DS. However, the values of $2T_m$ for 5-DS and as well as for 16-DS bound to apomembranes were lower as compared to those

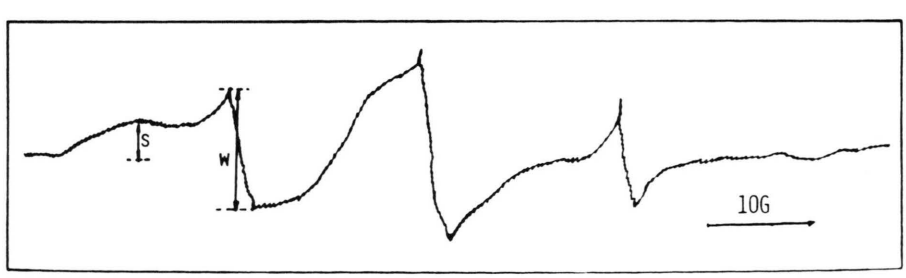

Fig. 4. ESR spectra of SSL label bound to purple membranes; W, weakly immobilized; S, strongly immobilized.

in PM. The difference of the values of hyperfine splitting parameter $2T_m$ observed between both membrane samples we interpreted as an indication that the motion of the spin labels was more restricted in apomembranes and that the lipid acyl groups in the interior of apomembranes were slightly less fluid than those in PM. Since the mobility of the spin label is determined by the order of the lipid bilayer, the reduced mobility of the lipid acyl chains could be attributed to a better packing of lipids due to rearrangement of protein or/and lipids after bleaching. In fact, the rearrangement of both protein and lipid components after conversion of BR into BO has been suggested in ref. [3]. Evidence for protein structural rearrangement accompanying the bleaching of BR has been given by fluorescence studies, showing that apomembranes were more easily penetrated by small ions [23]. It has also been demonstrated that bleached membranes were more leaky for protons, which support the view about more open configuration of BO [24]. The results presented in this study clearly show that the removal of the retinal from PM affects the dynamics of lipid bilayer and apomembranes are more rigid structure than those of native purple membranes. These alterations might be readily understood in term of the rearrangement of lipid bilayer induced by protein structural changes upon the removal of the retinal.

Acknowledgement

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