FLASH-INDUCED FAST CHANGE ON PURPLE MEMBRANE SURFACE DETECTED BY SPIN-LABEL METHOD

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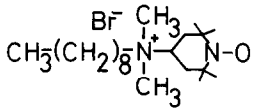
1. Introduction

Spin labels have provided useful information concerning structure and function related to biological membranes as well as phospholipid bilayer membranes. Charged spin labels with some hydrophobic tails may be used as an indicator of membrane surface potential. For example, partition of *N,N,*-dimethyl-*N*-nonyl-*N*-tempoyl-ammonium bromide (see formula) between phospholipid membrane and the surrounding aqueous phase was shown dependent on the membrane surface potential [1].



Here we have used the potential indicator spin label to follow fast photogenic surface potential change of purple membrane, and detected flash-induced ESR signal change in the membrane fragment suspension. This photogenic change was not observed in a bleached preparation, and reappeared on regeneration. The ESR signal change can be ascribed to the surface potential change of purple membrane resulting from the photoreaction of bacteriorhodopsin. Possible causes of this photogenic change are discussed in the light of its photoreaction kinetics.

2. Materials and methods

Potential indicator spin label *N,N,*-dimethyl-*N*-nonyl-*N*-tempoyl-ammonium bromide was synthesized as in [2], then converted into chloride form by application on an anion exchanger column chromatography. 12-Nitroxide stearic acid was synthesized as in [3], and used to measure the fluidity of the hydrophobic region of membranes.

Purple membrane fragments were prepared from *Halobacterium halobium* R_1 as in [4]. Some of them were bleached in an ice bath in the presence of 0.6 M hydroxylamine adjusted to pH 7.0 with sodium hydroxide [5] and washed with distilled water to remove hydroxylamine. Regeneration of purple membrane was performed by adding all-*trans* retinal in ethanol solution to bleached preparation [6]. The membrane fragments were suspended in water containing 1 mM potential indicator (pH 5.8) and then light-adapted by illumination with white light in an ice bath [7]. The ESR spectra were measured with an X-band spectrometer ME-2X (JEOLCO) at 2°C under dim light.

Fast flash-induced ESR signal change was measured as follows. White light flash with duration <1.7 ms was supplied with a strobo lamp (Kako F-4RS) set close to the ESR cavity, and focused on a sample tube with a convex lens. ESR signal at fixed field was accumulated using a spectrum computer JEC-EC-100 with 100 μs delay time after the trigger pulse firing the flash lamp. Trigger pulse was generated with a pulse programmer JEC-DP-1. Response time of the spectrometer was 1 ms. In most cases, 100 transients were accumulated with 40 s pulse interval time.

Absorption spectra of the membrane preparations were measured at 23°C with spectrophotometer Hitachi 323 after light adaptation, to determine the concentration of bacteriorhodopsin in preparations and to estimate the degree of bleaching or regenera-

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tion. An extinction coefficient of 63 000 at 570 nm [8] was used for light-adapted bacteriorhodopsin. The concentration of bacterioopsin in a bleached preparation was determined from comparison of A_{280} with that of bacteriorhodopsin in purple membrane.

3. Results

3.1. *Fast photogenic ESR signal change in light-adapted purple membrane fragment suspensions*

An ESR spectrum of the potential indicator spin label in light-adapted purple membrane fragment suspension is shown in fig.1. The spectrum consists of two components; the sharp triplet line arising from the labels in the aqueous phase, and the broad line from those in the membrane phase. Partition of the label between the two phases was affected by the ionic strength of the suspending medium. For example, the peak-height ratio of the two components at the low field, L/M (see fig.1), was increased lineary ~ 2.9-times by raising medium [salt] from 2.5–100 mM at constant pH. The dependence of the label partition on the ionic strength was well explained based on the theory of Gouy-Chapman for high potential (data not shown). This result showed that the partition was dependent on the membrane surface potential. The overall splitting value of the broad component was 65.9 G at 2°C. Strong immobilization of the polar head group of the label molecules may be probably due to electrostatic interactions with the negative groups on the purple membrane surface, since TEMPO-phosphatidylcholine incorporated in the purple membrane did not give such a rigid ESR spectrum (data not shown).

The ESR spectrum of 12-nitroxide stearic acid incorporated in the purple membrane fragments showed the rigid environments in the membrane hydrophobic region. The overall splitting value of the ESR spectrum was 60.9 G at 23°C. This value is close to 60.7 G obtained at 25°C using the same spin label [9].

To follow the flash-induced ESR signal change of the potential indicator, the magnetic field was fixed at the high-field peak position of the 'aqueous component' (see arrow in fig.1), since the peak height at this position is sensitive to the label distribution between the two phases [1]. Flash illumination caused a rapid decrease in the peak height as shown in fig.2. The half-time for the decrease was 4 ms (see inset). The peak-height then gradually recovered to the initial level with the half-time of 70 ms. When the flash-induced change was observed at the 'membrane component', M (at the low-field peak position, see fig.1), a transient increase of the peak-height and the following recovery were observed. These changes, therefore, indicate a flash-induced transient change of the label distribution; increase in the membrane phase and decrease in the aqueous phase of the label population. The initial rapid decrease of the aqueous component signal was found to be almost independent of suspending medium [salt] from 1 mM–1 M.

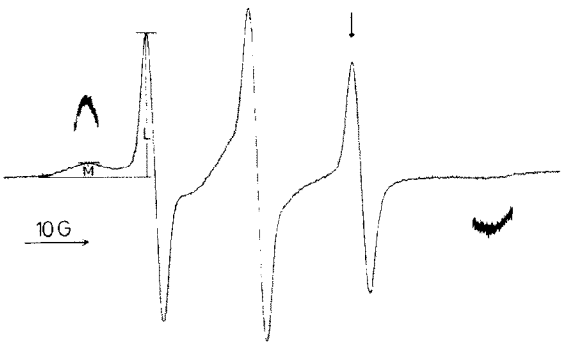

Fig.1. ESR spectrum of the potential indicator spin label in the aqueous suspension of light-adapted purple membrane fragments at 2°C. To know the overall splitting of the membrane component (see section 3), high-field and low-field peaks of the component were recorded with higher gain. Bacteriorhodopsin was 6.3 mg/ml.

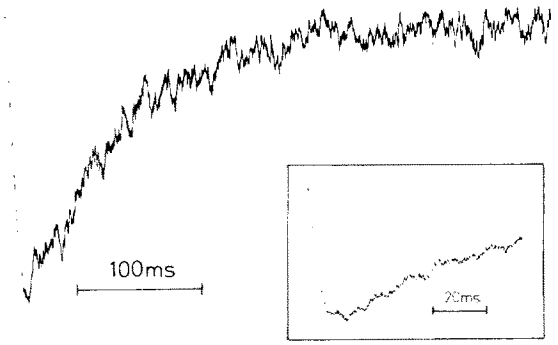

Fig.2. ESR signal change induced by flash illumination. Magnetic field was fixed at the position indicated by the arrow in fig.1, and 100 transients were accumulated. Signal was sampled for 650 ms on 8096 points in one transient. The inset shows the initial phase with sampling for 130 ms on 8096 points.

Flash-induced ESR signal change was also observed with a neutral spin label 4,4′,-dimethyloxazolidine-*N*-oxyl derivative of diisobuthylketone. This spin label also partitioned between the aqueous and the membrane phases in the purple membrane suspension. Flash illumination caused some decrease of the aqueous phase signal, but the decrease was much smaller and slower than that for the potential indicator spin label.

3.2. *No photogenic ESR signal change in a bleached membrane preparation*

An ESR spectrum of the potential indicator in the suspension of bleached membrane fragments is shown in fig.3a. The membrane component was much larger than that in the purple membrane suspension. This drastic spectral change is not due to the difference in the membrane concentration, but reflects more partition in the membrane phase.

The overall splitting of the membrane component was 67.3 G at 2°C and that of the ESR spectrum of 12-nitroxide stearic acid label was 62.8 G at 23°C. These values were slightly larger than those for the purple membrane fragments.

No photogenic ESR signal change was observed in the bleached membrane preparation (fig.3b).

3.3. *Reappearance of photogenic ESR signal change on regeneration of purple membrane*

The increased membrane component of the bleached preparation became smaller on the regeneration of purple membrane from bleached membrane (fig.4a). The increased overall splitting was also decreased to 65.1 G on regeneration. However, the recovery of the spectrum was not complete. For example, the peak-height ratio of the membrane component to the aqueous component (L/M) was 9.1, 1.9 and 3.4 for the intact, the bleached and the regenerated preparations, respectively. If we assume that the regenerated preparation consists of the bleached and the regenerated membrane (purple membrane) fragments, the fraction of the regenerated membrane is calculated as 0.47 using the peak-height ratio. This value is close to that of 0.43 estimated from the absorption spectra.

The regenerated preparation showed a definite photogenic ESR signal change, similar in time course to that for the intact preparation (fig. 4b).

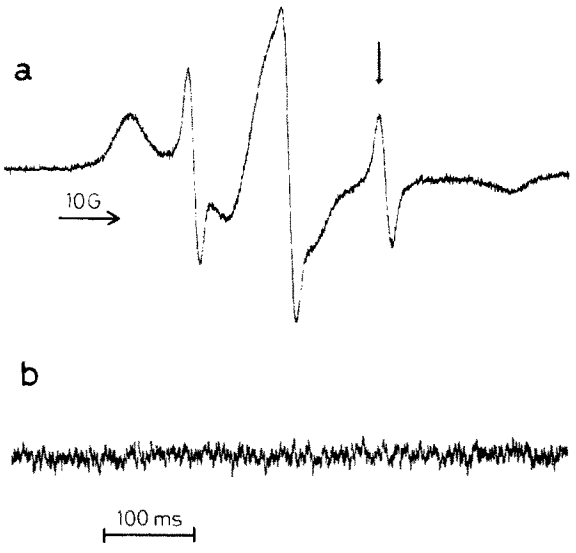

Fig.3. (a) ESR spectrum of the potential indicator spin label in the aqueous suspension of bleached membrane fragments at 2°C. Bacterioopsin was 5.8 mg/ml. (b) No photogenic ESR signal change was observed on flash illumination. Magnetic field was fixed at the position indicated by the arrow in fig.3(a) and 100 transients were accumulated under the same conditions as that for fig.2.

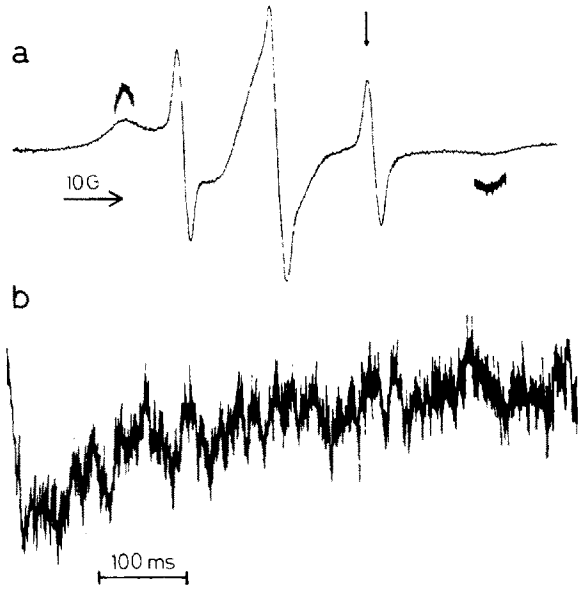

Fig.4. (a) ESR spectrum of the potential indicator spin label in the aqueous suspension of regenerated purple membrane fragments at 2°C. Bacteriorhodopsin is the same as that for fig.3(a). (b) Photogenic ESR signal change observed at the magnetic field position indicated by the arrow in fig.4(a). 100 transients were also accumulated.

4. Discussion

The flash-induced change observed in the ESR spectrum of the potential indicator is due to the photoreaction initiated by absorption of light by *trans*-bacteriorhodopsin. Disappearance and reappearance of the spectral change in the bleached and the regenerated membrane preparations, respectively, supports the assignment of the signal change. The spin label itself has a weak absorption in the visible region with maximum at 430 nm, but was not affected by the flash illumination.

The fast flash-induced increase of the label partition into the membrane phase may be caused by decrease of the membrane surface potential or by increase of the membrane fluidity, or both on flash illumination. Much smaller increase of partitioning of the neutral spin label and its slower time course indicate the membrane surface potential change as the main origin of the photogenic decrease. However, the flash-induced fast increase of the label partition was almost independent of the ionic strength of the surrounding medium. A similar result [10] was ascribed to a greater change in surface charge density at the higher ionic strength. Experiments with negatively-charged spin labels should be more conclusive.

The transient decrease of the membrane potential agrees with the proton release out of the membrane on photoreception [11]. The following recovery of the potential will be caused by re-adsorption of proton. The observed transient change may, therefore, be related to *meta*-intermediate, known as a deprotonated intermediate in the photoreaction cycle [12]. The half-time for the formation of *meta*-intermediate has been reported as 0.32 ms at 0°C in water [13], which is much smaller than that of the observed half-time (4 ms) for the transient ESR signal change. This difference may be at least partly due to slower response of our instrument and long flash duration. Time required for the partitioning of the spin-label may also be the cause of the difference. Of course, there is a possibility that the difference reflects a time lag between the formation of *meta*-intermediate and proton release [14].

The signal change observed with the neutral spin-label indicates transient fluidizing of the purple membrane during the photoreaction of bacteriorhodopsin. This may be due to some conformational change of the bacteriorhodopsin molecules in the photochemical reaction.

The relation between the photogenic change and proton translocation kinetics is being studied by improving time resolution of observation system.

Acknowledgements

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