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Culture Temperature Affects the Molecular Motion of Bacteriorhodopsin within the Purple Membrane

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We measured the absorption anisotropies of bacteriorhodopsin (bR) within a purple membrane suspension after photo-excitation in the millisecond time range. The purple membranes used were isolated from *Halobacterium salinarium* grown at three different culture temperatures, 37.0, 43.0 and 47.5 °C. For the membranes from the 37.0 °C culture, the observed anisotropies at wavelengths of 410, 570 and 680 nm showed almost the same slow decay. The slow decaying of the anisotropies originated from the rotation of the membrane itself. Using the membranes from the 43.0 and 47.5 °C culture, however, we found that the anisotropy change varied at each wavelength measured. In these cases, it is shown from detailed data analysis that 1) the rotational motion of photo-intermediates within the membrane is more restricted than that of non-excited bR and 2) the distorted arrangements of the proteins within the membrane remain, even after photo-intermediates return to ground-state bR. This restricted motion is probably caused by the conformational changes in photo-intermediates, which prevent the rotation of the monomer protein and/or lead photo-intermediates to bind with neighboring proteins.

Key words bacteriorhodopsin; absorption anisotropy; protein molecular motion; culture temperature effect

The purple membrane of *Halobacterium salinarium* (formerly *halobium*) contains a single protein, bacteriorhodopsin (bR),[1] of which the structure is similar to that of the visual pigment of animal eyes. Both proteins include a retinal chromophore, which absorbs light and triggers photo-reactions. However, the functions of these proteins are different. bR shows a cyclic photo-reaction[2] and serves as an energy transducing pigment. In this cyclic photo-reaction, bR transforms several intermediates in sequence, then returns to ground-state bR. During this photochemical cycle, bR translocates a proton from inside to outside the cell.

In the purple membrane, bR molecules are arranged in trimmers, and the trimmers form the hexagonal lattice.[3] Due to such a crystalline structure, it is expected that the molecular motion of bR is severely restricted. Thus, whether or not the protein moves within the membrane is in question. To detect the molecular motion, the use of an absorption anisotropy method has been widely accepted. Upon the excitation of bR with a polarized actinic flash, bR molecules whose absorption dipoles are parallel or near parallel to the polarized direction of the actinic flash are selectively excited and undergo the photochemical cycle. Thus, the excited bR molecules have similar orientations just after flashing. When the bR molecules make a rotational motion, these orientations of the excited molecules become random and absorption anisotropy decreases. Using this method, several investigators reported the complete immobilization of the protein within the membrane.[4-8] They observed the slow decay of the anisotropy in the purple membrane suspension, and attributed the slow decay to the rotational motion of the membrane itself.

On the other hand, a few investigators observed wavelength-dependent anisotropy changes.[9-11] Photo-intermediates have their own maximum wavelengths. When the time-course of anisotropy change varies in photo-intermediates and ground-state bR, wavelength-dependent anisotropy changes should be observed. In these reports, they suggested that the wavelength-dependent anisotropy changes originated from the rotational motion of the protein.

As seen above, the reported results are contradictory and controversial. In this work, we observed both wavelength-dependent and -independent anisotropy changes using the purple membranes isolated from cells grown at different temperatures. The wavelength-dependent changes in the anisotropies appeared with a rise in the culture temperature. We will report the effect of culture temperature on anisotropy changes, and discuss the motion of the protein within the membrane.

Materials and Methods

Materials *Halobacterium salinarium* strain s9 was grown at 37.0, 43.0 and 47.5 °C. The purple membranes were isolated from the cells according to the standard method.[12] All samples were suspensions of the purple membranes in a buffer solution. The buffer solution was sodium phosphate, 10 mM, pH 6.9. The absorbance values at 570 nm of the samples were adjusted to be about 1.0.

Measurement of Absorption Anisotropy The actinic flash (7 ns, 532 nm) was the second-harmonic of the fundamental beam of a Q-switched Nd-YAG laser (Quanta-Ray, DCR-2). To polarize the actinic flash vertically, a Glan laser polarizer (Optics for Research, PLU-10) was placed just in front of the sample. Two monochromators, placed in back of the monitoring light source and in front of the photomultiplier (Hamamatsu, R2949), were used to select the measuring wavelength and to eliminate the scattering light of the flash from the sample. The monitoring light was polarized by two sheet polarizers placed in front and back of the sample. Rotating the sheet polarizers, the polarized direction of the monitoring light was changed. The absorption changes at λ nm, $\Delta A_\lambda^{\parallel}(t)$ and $\Delta A_\lambda^{\perp}(t)$, were obtained with the monitoring light polarized parallel and perpendicular to the polarized direction of the actinic flash. Using $\Delta A_\lambda^{\parallel}(t)$ and $\Delta A_\lambda^{\perp}(t)$, the absorption anisotropies (r_λ) were calculated using the following equation:

$$r_\lambda(t)=\frac{\Delta A_\lambda^{\parallel}(t)-\Delta A_\lambda^{\perp}(t)}{\Delta A_\lambda^{\parallel}(t)+2\Delta A_\lambda^{\perp}(t)} \quad (1)$$

The energy of the actinic flash was adjusted to activate about 5% of

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all bR molecules. Before each measurement, samples were light-adapted with exposure to a 150-W lamp. All measurements were performed at 20.0 °C.

Results

Figure 1 shows the flash-induced absorption anisotropies of purple membrane suspension which was isolated from cells grown at 37.0 °C. Lozier *et al.* showed that the photochemical cycle includes absorption changes corresponding to the formation and decay of photo-intermediates.[2] In the millisecond time range, the measurable species are M-, O-intermediates and ground-state bR. Under our experimental conditions, the photochemical cycle is expressed as "ground-state bR→M→O→ground-state bR" (the arrows indicate the photochemical process). The maximum wavelengths in the absorption spectra of these species are different. Thus, we selected three measuring wavelengths of 410, 570 and 680 nm, at which M-intermediate, ground-state bR and O-intermediate were mainly monitored, respectively. The measured anisotropies at these three wavelengths decreased at similar rates. This fact shows that ground-state bR and photo-intermediates undergo almost the same motion. The slow decay of the anisotropies is similar to that of previously reported results, in which this slow decay was explained by the rotational motion of the membrane itself.[5,8]

For the purple membranes obtained from the cells at 43.0 and 47.5 °C, however, we measured wavelength- and culture temperature-dependent anisotropy changes. As shown in Fig. 2, characteristic anisotropy changes were caused at each wavelength measured. At 410 nm, the anisotropies for the 43.0 and 47.5 °C culture increased with time. At 680 nm, for the 47.5 °C culture, the anisotropy also increased. On the other hand, at 570 nm, the anisotropies for the 43.0 and 47.5 °C culture decreased and took negative values. The wavelength-dependent anisotropies indicate that the motion of absorption dipole changes during the photochemical cycle.

The absorption spectra of photo-intermediates and ground-state bR overlap each other.[13,14] The measured anisotropy change implies the motion of several species. In the millisecond time range, the species which appeared

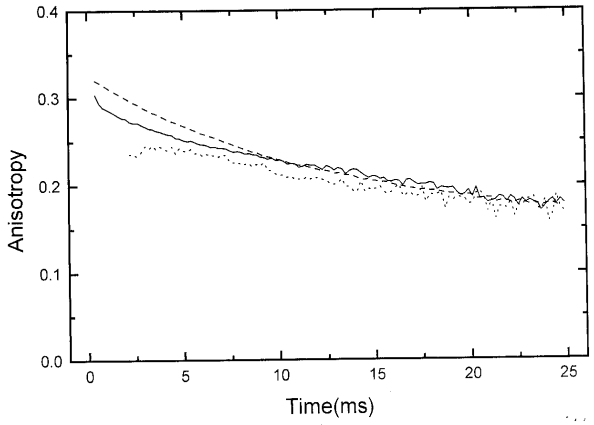

Fig. 1. Absorption Anisotropies of the Purple Membrane, Which was Isolated from Cells Grown in a 37.0 °C Culture

The anisotropy measurements were performed at 20.0 °C. The wavelengths measured are 410 (——), 570 (– – –) and 680 nm (------). The anisotropies at the three wavelengths decay at almost the same rates.

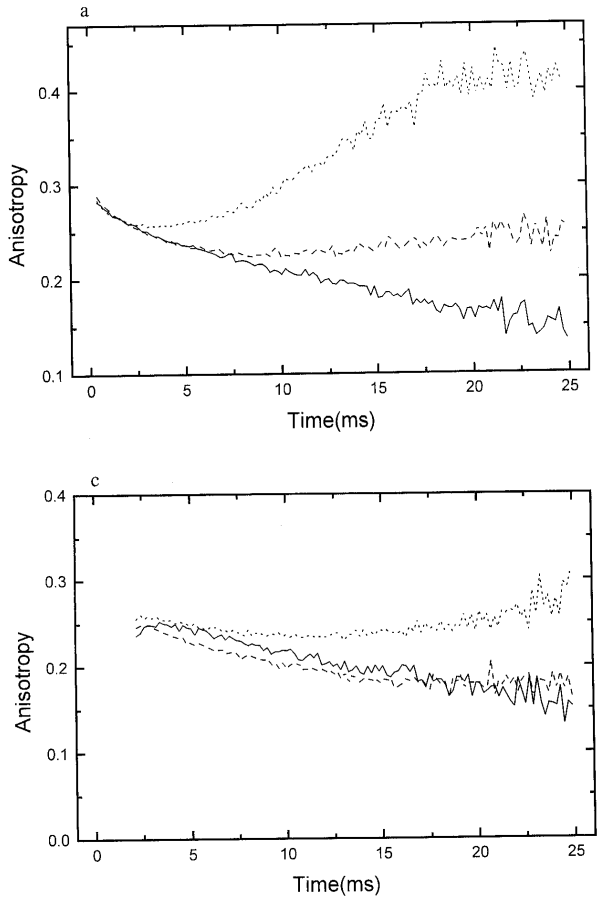

Fig. 2. Absorption Anisotropies of the Purple Membranes Isolated from Three Kinds of the Cells

The cells were grown at 37.0 °C (——), 43.0 °C (– – –) and 47.5 °C (------) culture. The anisotropy measurements were performed at 20.0 °C. (a) Data obtained at 410 nm. For the purple membranes from the 43.0 and 47.5 °C culture, the anisotropies increase with time. (b) Data at 570 nm. For the purple membranes from the 43.0 and 47.5 °C culture, the decay of anisotropies becomes rapid and the anisotropies take negative value. (c) Data at 680 nm. For the purple membrane from the 47.5 °C culture, the anisotropy increases with time.

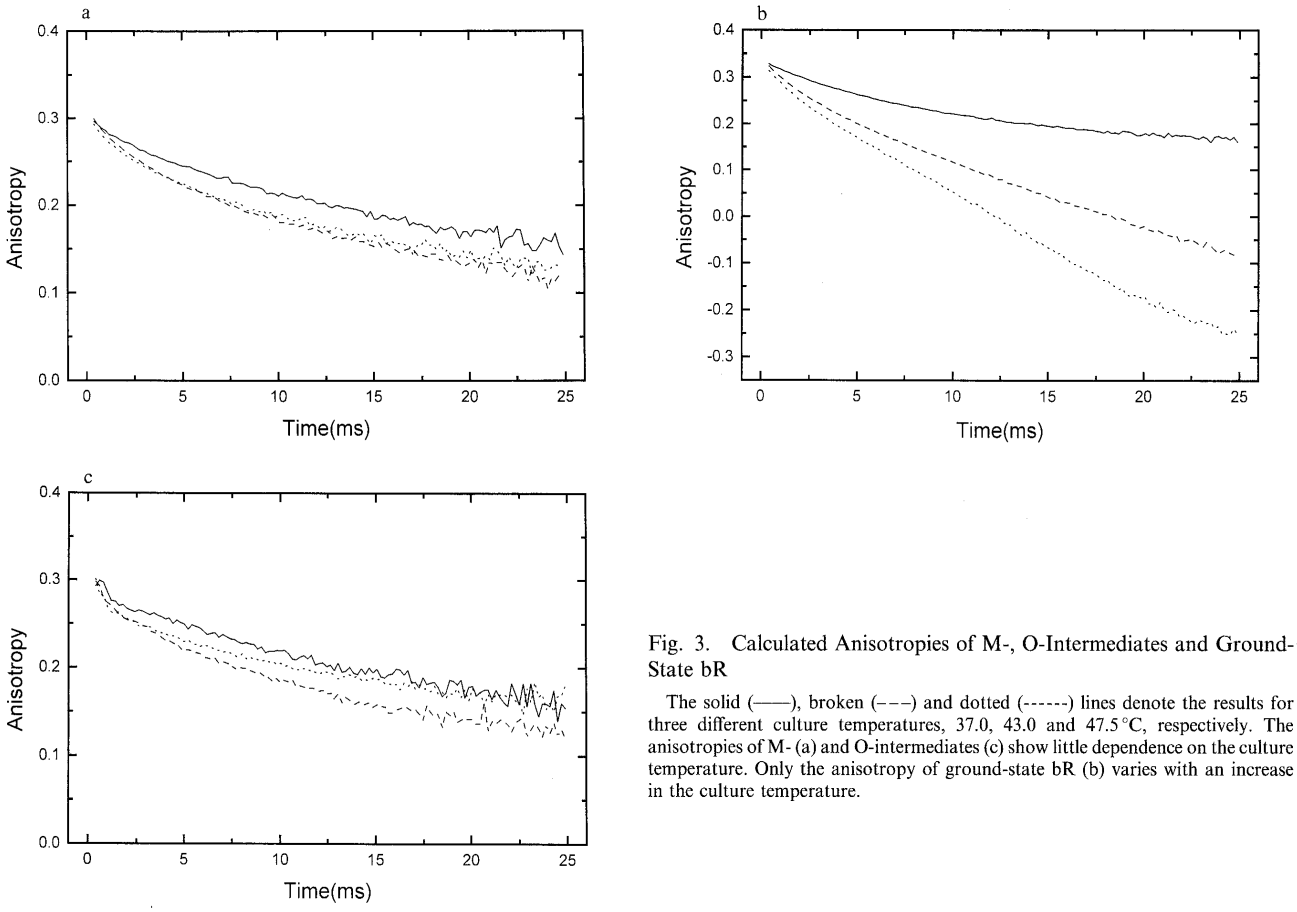

Fig. 3. Calculated Anisotropies of M-, O-Intermediates and Ground-State bR

The solid (——), broken (– – –) and dotted (------) lines denote the results for three different culture temperatures, 37.0, 43.0 and 47.5 °C, respectively. The anisotropies of M- (a) and O-intermediates (c) show little dependence on the culture temperature. Only the anisotropy of ground-state bR (b) varies with an increase in the culture temperature.

are M-, N-, O-intermediates and ground-state bR. Under our measuring conditions, in which the pH was kept at 6.9, the amount of accumulation of N-intermediate is small.[15] Thus, we took into consideration the absorption overlapping from M-, O-intermediates and ground-state bR, and calculated the anisotropies of these species. The absorption change (ΔA_λ) and anisotropy (r_λ) at λ nm are expressed by the following equations.

$$\Delta A_\lambda(t)=\alpha_\lambda \Delta A^{M}_{410}(t)+\beta_\lambda \Delta A^{bR}_{570}(t)+\gamma_\lambda \Delta A^{O}_{680}(t) \quad (2)$$

$$r_\lambda(t)=\frac{\alpha_\lambda \Delta A^{M}_{410}(t) r^{M}(t)+\beta_\lambda \Delta A^{bR}_{570}(t) r^{bR}(t)+\gamma_\lambda \Delta A^{O}_{680}(t) r^{O}(t)}{\Delta A_\lambda(t)} \quad (3)$$

where

$$\alpha_\lambda=\frac{\varepsilon^{M}_\lambda}{\varepsilon^{M}_{410}},\quad \beta_\lambda=\frac{\varepsilon^{bR}_\lambda}{\varepsilon^{bR}_{570}},\quad \gamma_\lambda=\frac{\varepsilon^{O}_\lambda}{\varepsilon^{O}_{680}}$$

Here, $\Delta A^{\Omega}_\lambda$ and r^{Ω} (Ω=M, O, bR) are the absorption change induced by Ω species and the anisotropy attributed exactly to the Ω species, respectively. The term of $\varepsilon^{\Omega}_\lambda$ is the extinction coefficient of the Ω species at λ nm. Using Eqs. 2 and 3, we calculated r^{Ω} and plotted these in Fig. 3. The values of $\varepsilon^{\Omega}_\lambda$ were determined according to the previously reported absorption spectra.[13,14] The values used of α_{570}, α_{680}, β_{410}, β_{680}, γ_{410} and γ_{570} were 0, 0, 0.22, 0.03, 0.25 and 0.75, respectively. As shown in Fig. 3, the anisotropy changes of M- and O-intermediates showed similar decays and did not display remarkable differences with respect to the culture temperature. On the other hand, with the rise of the culture temperature, the decay of the anisotropy of ground-state bR became rapid. Therefore, we conclude that culture temperature affects only the motion of retinal in ground-state bR.

Discussion

The induced decay of r^{bR} by a rise in the culture temperature should originate from the wobbling motion of the chromophore inside the protein and/or from the rotation of the whole protein within the membrane. The retinal chromophore is surrounded by the peptide chains. In this condition, the wobbling motion of the chromophore should be constrained, and be related to the motion of the peptide chains. The motion of the peptide chains usually occurs from the picosecond to nanosecond time range.[16] Consequently, the wobbling motion of the chromophore should make little contribution to observed anisotropy decay in this work. Thus, the rotational motion of whole protein is considered to be the main origin for the motion of the chromophore within the membrane.

In the normal rotational diffusion of a chromophore, the minimum value of anisotropy is zero, because the orientation of the chromophore finally becomes random. As shown in Fig. 3, however, the anisotropy of ground-state bR took a negative value. The flash excitation depletes the population of ground-state bR, and so the absorption change induced by ground-state bR is negative. Thus, the anisotropy of ground-state bR is that of imaginary 'disappearing' molecules. The shift in anisotropy, from positive to negative, corresponds to a shift in the horizontally polarized angular distribution of absorption dipoles in ground-state bR to a vertically

polarized one.

To understand why the anisotropy of ground-state bR took a negative value, we must recall that ground-state bR implies two species, non-excited bR and 'returned' bR. Here, 'returned' bR means ground-state bR that had been activated by flash-excitation and had finished the photochemical cycle. According to the situation, whether the angular distribution of absorption dipoles in these two species depolarizes at the same rates or not, the explanation for the negative value of the anisotropy divides into the following two cases. (Case 1): Both non-excited bR and 'returned' bR rotate, and have the same average angles of rotation exceeding 54.7 degrees. (Case 2): The angular distribution of absorption dipoles in non-excited bR depolarizes faster than that in 'returned' bR. In the first case, a serious problem arises. The average of rotational angle in this case is larger than that induced by the normal Brownian motion of the molecules. When the molecules rotate by their normal Brownian motion, the anisotropy finally reaches zero. This process is equivalent to a case in which all molecules rotate with the same angles of 54.7 degrees. Therefore, in the first case, rotations with a large average angle above 54.7 degrees have to be induced by a large conformational change in the photo-excited molecules. If this case were correct, the induced large rotation of the neighboring non-excited molecules would propagate throughout the membrane. It is not likely that the propagating rotations are caused by only 5% photo-excitation of all molecules. Therefore, we must choose the second case as the reason for the negative anisotropy values.

The actinic flash is polarized vertically. Thus, immediately after the flash excitation, the angular distributions of the absorption dipoles in M-intermediate and non-excited bR are polarized vertically and horizontally, respectively. The rotations of photo-intermediates within the membrane are considered to be negligible (Fig. 3a, c). Thus, the rotations of non-excited bR within the membrane lead to the faster depolarization of the angular distribution of non-excited bR than that of photo-intermediates. In this situation, when 'returned' bR maintains the vertically polarized distribution of photo-intermediates, the angular distribution of all ground-state bR can be polarized vertically. The rotation of non-excited bR can be attributed to the normal Brownian motion of non-excited bR.

Consequently, our experimental results are explained by the measurable mobility of non-excited bR within the membrane being induced by an increase in culture temperature. However, our results also indicate that the motion of photo-intermediates is still restricted. These facts express an important characteristic of bR protein. The mechanism of restricting the motion of photo-intermediates must be derived from conformational changes in photo-intermediates. The changed conformation is considered to prevent the rotation of the monomer protein and/or to make photo-intermediates bind with neighboring proteins. The conformational change in the inner part of the protein around the retinal molecule cannot influence the rotation of the whole protein. Thus, the conformation at the outer part of the protein should alter.

The primary structure of the protein should not depend on the culture temperature. The culture temperature is considered to affect the lipid composition and/or the amount of the protein in the membrane. Such perturbation of membranes may influence the lipid–protein and the protein–protein interaction. In this work, the increase in culture temperature induces the rotational motion of ground-state bR within the membrane and makes the restricted motion of photo-intermediates become measurable.

For halorhodopsin (hR), the interaction between hR and carotenoids was reported.[17] The photo-excitation of hR leads to an absorption change of carotenoid that is associated with hR. The decay of the carotenoid signal is slower than that of the hR photochemical cycle. This suggests that the recovery of the changed conformation in hR should be slower than that of the absorption change of the hR photocycle. The observed negative anisotropy of ground-state bR in this work indicates that the distorted arrangement of the proteins within the membrane remains, even after photo-intermediates return to ground-state bR. This means that the changed conformations in photo-intermediates of bR remain after the absorption change of the photochemical cycle has been finished, as is the case with hR.

The visual pigment of animal eyes, rhodopsin, causes a change of its outward conformation by photo-excitation. The signal transducing protein recognizes the changed conformation in rhodopsin. Therefore, our observed conformational change at the outer part of photo-intermediates and/or their bindings with neighboring proteins are considered to be an intrinsic property of the retinylidene proteins

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