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# Between the ground- and M-state of bacteriorhodopsin the retinal transition dipole moment tilts out of the plane of the membrane by only 3°

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The orientation of the transition dipole moments in the ground state and the M-intermediate of bacteriorhodopsin were determined by time-resolved and steady-state polarized absorption spectroscopy on samples of oriented immobilized purple membranes. The angle between the transition dipole moment and the membrane normal decreases from 66.8±0.5° in the all-*trans* ground state to 64.1±0.8° in the 13-*cis* M-state. The light-induced isomerization of the chromophore is thus accompanied by an orientational change of only about 3° out of the plane of the membrane. The absorption anisotropy at 410 nm remains constant over more than 4 decades of time covering both the rise and decay of M. Conformational changes accompanying a sequential $M_1 \rightarrow M_2$ transition thus do not affect the chromophore orientation.

Bacteriorhodopsin; Flash spectroscopy; Linear dichroism; Isomerization; Retinal

## 1. INTRODUCTION

In the course of its photocycle the chromophore of bacteriorhodopsin (bR) isomerizes around the 13–14 double bond. Whereas in the light-adapted ground state the chromophore is all-*trans*, the intermediates K, L, M and N are 13-*cis* [1]. The isomerization of the chromophore is expected to be associated with changes in the orientation of the electronic transition dipole moment. Time-resolved optical absorption spectroscopy with polarized light offers an attractive method to monitor the isomerization in real time by measuring the angle $\theta$ between the transition dipole moment and the membrane normal. In previous work in this area isotropic gels or suspensions were used [2,3]. With such samples the observed absorption anisotropy of the intermediate M is, however, quite insensitive to changes in angle, since it is given by 0.4 $P_2(\cos\theta_{MO})$. $P_2$ is the second Legendre polynomial and $\theta_{MO}$ is the change in angle of the transition dipole moment between the ground state (0) and the intermediate M (see Fig. 1). Thus if for instance $\theta_{MO} = 10°$, the anisotropy in the intermediate differs by only 4.7% from that in the ground state (0.4). The anisotropy observed in isotropic samples for the M intermediate was the same as in the ground state, indicating that the angular change is small [3]. Photoselection measurements with FTIR also showed that the direction of the C=C chain of the chromophore does not significantly change in the bR-to-M transition [4]. A more sensitive method is clearly required. We have measured the time-resolved absorption anisotropy in samples of purple membranes that were oriented in a magnetic field and subsequentely immobilized in a gel thus eliminating the decay of the anisotropy due to the rotational diffusion of the membranes. The orientation, even if non-perfect, leads to a wealth of additional information as is well known from the analogous case of emission spectroscopy [5]. This is due to the very different photoselection in anisotropic samples. The main advantage of oriented samples is that one measures $P_2$ of the angle between the transition dipole moment of an intermediate and the membrane normal. Since these angles are expected to be close to that of the ground state (~67°), such measurements are very sensitive. A change of only 1° around 67° already leads to a change in anisotropy of 7.6%.

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## 2. MATERIALS AND METHODS

Purple membranes (15 μM bR in 25 mM phosphate buffer, pH 7) were oriented and immobilized in a 10% polyacrylamide gel in a standard 4×10 mm quartz cuvette placed in a 13 T magnetic field. The orientation was monitored by the increase in birefringence. After completion of the polymerization, the field was turned off and the birefringence remained constant. Purple membranes orient in a magnetic field with their normals parallel to the field [6] (see Fig. 1). One important advantage of gels over oriented films is that water content, pH, and salt concentration are well defined. Flash spectroscopy was carried out as described [7].

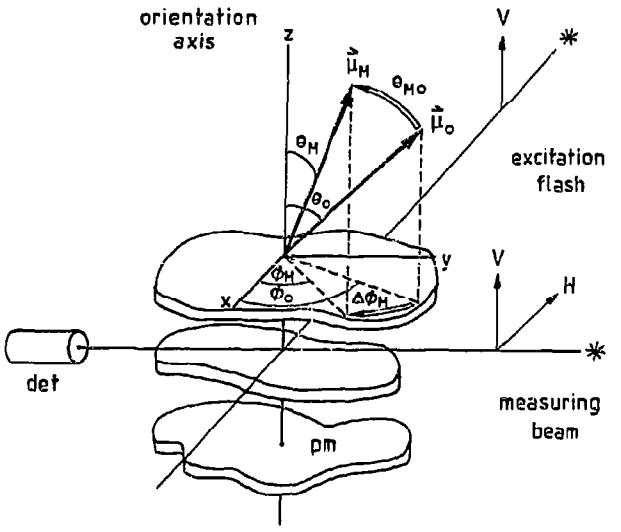


Fig. 1. Geometry of polarized flash experiments with purple membranes (pm) that are oriented and immobilized in a gel. The orientation axis coincides with the magnetic field direction. $\vec{\mu}_0$ and $\vec{\mu}_M$ are the transition dipole moments in ground (0) and M-state respectively. Their spherical polar coordinates $\Theta_0$, $\Phi_0$, $\Theta_M$, $\Phi_M$ as well as $\Delta\Phi_M$ and $\Theta_{MO}$ are defined with respect to a membrane fixed coordinate system. In the case of perfect orientation the transition dipole moments are on the surface of a cone with opening angle $\Theta_0$ and saturation effects do not occur with vertical excitation.

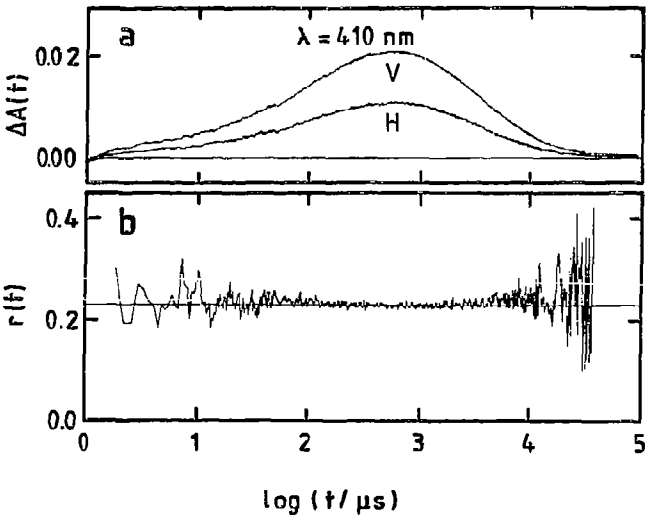


Fig. 2. (a) Flash-induced absorbance changes $\Delta A_V$ and $\Delta A_H$ at 410 nm with vertical excitation and with polarizer in the measuring beam vertical and horizontal respectively. Average over 200 low intensity 10 ns flashes at 580 nm. The horizontal coordinate is the time after the flash in logarithmic units. The sample was in the light-adapted state. (b) Absorption anisotropy $r_{410}(t)$ calculated from (a). The signal-to-noise ratio is poor for very short and very long times since $\Delta A$ approaches zero. The horizontal line through the data represents the constant average anisotropy of 0.23.

## 3. RESULTS

The time-resolved and steady-state absorption anisotropy measurements were carried out in the geometry of Fig. 1. The excitation flash at 580 nm is polarized parallel to the orientation axis. From the absorbance changes $\Delta A$ with the polarizer in the measuring beam at wavelength $\lambda$ set parallel (V) and perpendicular (H) to the excitation polarization, the anisotropy $r_\lambda(t)$ is calculated:

$$r_\lambda(t) = \frac{\Delta A_V - \Delta A_H}{\Delta A_V + 2\Delta A_H} \quad (1)$$

With an isotropic immobilized sample and a measuring wavelength of 550 nm (depletion signal) the anisotropy should be 0.40 at low flash intensity. Under these conditions we obtained a time-independent value of 0.39±0.01 (data not shown), proving that the anisotropy equipment is operating correctly. Fig. 2a shows the absorbance changes at 410 nm due to the rise and decay of the M-intermediate for oriented immobilized membranes over 5 orders of magnitude in time from 1 μs to 0.1 s. Fig. 2b shows that the corresponding anisotropy is constant in time due to the elimination of the membrane rotation. This allows the extraction of a very accurate value for the anisotropy in this particular sample of 0.23. In the same way data were obtained for the depletion signal at 550 nm, again showing a constant $r(t)$ of 0.19 over the whole time range (data not shown). In addition the steady-state anisotropy $r_s$ at 550 nm was determined to be −0.157. These 3 experiments were repeated 6 times with different samples and with different parts of the gels. The 3 experimental numbers $r_{550}(t)$, $r_{410}(t)$ and $r_s$ are related to the angles $\Theta_0$, $\Theta_M$ and the width $\Theta_{1/2}$ of the orientational distribution of the membranes according to:

$$r_s = S_2 P_2(\cos\Theta_0) \quad (2)$$

$$r_{550}(t) = \frac{2/5 + 11/7\ S_2P_2(\cos\Theta_0) + 36/35\ S_4P_4(\cos\Theta_0)}{1 + 2\ S_2P_2(\cos\Theta_0)} \quad (3)$$

$$r_{410}(t) = [S_2P_2(\cos\Theta_M) + 2\ P_2(\cos\Theta_0)\ P_2(\cos\Theta_M)(1/5 + 2/7\ S_2 + 18/35\ S_4) + 6\cos\Theta_0\cos\Theta_M\sin\Theta_0\sin\Theta_M\cos\Delta\Phi_M(1/5 + 1/7\ S_2 - 12/35\ S_4) + 3/2\sin^2\Theta_0\sin^2\Theta_M\cos2\Delta\Phi_M(1/5 - 2/7\ S_2 + 3/35\ S_4)]/(1 + 2\ S_2P_2(\cos\Theta_0)) \quad (4)$$

The details of the derivation of (3) and (4) will be presented elsewhere. Briefly the results can be obtained by adapting the analysis for emission anisotropy [5] to the case of absorption anisotropy. $S_2$ and $S_4$ are the order parameters of the orientational distribution of the purple membranes in the gel and are a measure of the quality of the orientation [8]. For perfect orientation both have a value of 1.0 and (2), (3) and (4) reduce to the simple and intuitively obvious results: $r_s = P_2(\cos\Theta_0)$, $r_{550}(t) = P_2(\cos\Theta_0)$ and $r_{410}(t) = P_2(\cos\Theta_M)$. In this case the angles $\Theta_0$ and $\Theta_M$ can be obtained directly and with high accuracy. Since in a 13 T magnetic field

the orientation is good but not perfect, the order parameters have to be taken into account. The orientational distribution $f(\Theta)$ and anisotropy of the magnetic susceptibility $\Delta\chi$ of purple membranes are well known [6] ($\Theta$ is the angle between the membrane normal and the field, $\Theta_{1/2}$ is the halfwidth of the distribution):

$$f(\Theta) = \exp[-\ln 2 \sin^2\Theta/\sin^2 \Theta_{1/2}] \quad (5)$$

with

$$\sin^2\Theta_{1/2} = \frac{2kT\ln 2}{\Delta\chi \cdot A \cdot d \cdot H^2} \quad (6)$$

$\Theta_{1/2}$ is determined by the known values of $\Delta\chi$, the magnetic field $H$, the membrane area $A$ and thickness $d$. The order parameters $S_2$ and $S_4$ are integrals of the product of $f(\Theta)$ with $P_2$ and $P_4$ [8], are thus simple functions of the halfwidth $\Theta_{1/2}$ and can be easily calculated. From the two experimental numbers $r_s$ and $r_{550}(t)$ for the ground state the two numbers $\Theta_0$ and $\Theta_{1/2}$ are extracted from (2) and (3) in a unique way. The average values from 6 independent experiments are $\Theta_0 = 66.8\pm0.5°$ and $\Theta_{1/2} = 23.3°$. The value of $\Theta_0$ is in excellent agreement with a large number of steady-state linear dichroism measurements [2,9]. From (6) we obtain with $\Theta_{1/2} = 23.3°$, $\Delta\chi = 3.5 \cdot \text{erg cm}^{-3} \cdot \text{T}^{-2}$ [6], $H = 13\text{T}$, $d = 49\text{Å}$ a value for $A$ of 0.13 $(\mu\text{m})^2$, in excellent agreement with electron microscope observations of the average membrane area in this preparation. The theoretical formalism employed thus leads to correct results for the ground state. $\Theta_{1/2}$ fixes both $S_2$ and $S_4$. The values for $\Theta_0$, $S_2$ and $S_4$ are now inserted into (4) to obtain $\Theta_M$ from $r_{410}(t)$. At first sight this seems impossible since (4) depends on both $\Theta_M$ and $\Delta\Phi_M = \Phi_M - \Phi_0$ (see Fig. 1). However measurements of $r_{410}(t)$ in isotropic samples [3], which we have confirmed and which show that within experimental error $r$ is the same at 550 and 410 nm, yield an upper limit of 10° for the total angular change $\Theta_{M0}$ between ground- and M-state. Therefore $\Delta\Phi_M$ is also less than 10°. Moreover in oriented samples $r_{410}(t)$ is very insensitive to the exact value of $\Delta\Phi_M$ within this range, since the $\Delta\Phi_M$-dependence of (4) resides in terms with cos $\Delta\Phi_M$. $\Theta_M$ was therefore calculated from (4) with $\Delta\Phi_M$ set at the two extreme values of 10° and 0°. For these 2 cases $\Theta_M = 63.8°$ and 64.3° and $\Theta_M - \Theta_0 = -3.0°\pm1.0°$ and $-2.5°\pm1.0°$ respectively, demonstrating the lack of sensitivity on $\Delta\Phi_M$. The angle $\Theta_M$ is thus between 1.5 and 4.0° smaller than $\Theta_0$ in the ground state, with the most likely difference around 3°. The average value of $\Theta_M$ is 64.1°.

## 4. DISCUSSION

We have developed a method to accurately measure the change in transition dipole moment orientation of the chromophore of bR that is applicable to all intermediates. The small change of −3° in M is not unexpected in view of the results with isotropic samples. Small changes of less than 11° were also observed with rhodopsin, using steady-state polarized spectroscopy [10].

The position of the chromophore within bR is known from neutron diffraction experiments with selectively deuterated retinals [11–13]. The plane of the polyene chain is perpendicular to the membrane [14] with the 9- and 13-methyl groups pointing towards the cytoplasm [13,15]. Recently it was shown by neutron diffraction that the $\beta$-ionone ring has the same in-plane position in ground- and M-state, but that in M the Schiff base end of the chromophore is displaced by about 1 Å roughly in the direction of the $\beta$-ionone ring (Haußß, Dencher, Büldt and Heyn, in preparation). These measurements also exclude a change in $\Phi$ of more than 10°, supporting our data analysis. The decreased $\Theta$ angle of the transition dipole moment in M is consistent with the shortened length of the projected chromophore observed by neutron diffraction. With the $\beta$-ionone ring staying fixed, the 13-*cis* chromophore in M is probably accommodated by a rearrangement of the lysine-216 side chain. An increase in $\Theta$ of perhaps 15° might have been expected if the isomerization only involved the atoms near the Schiff base beyond $C_{13}$ and if the polyene plane remained perpendicular to the plane of the membrane in M. To compensate for this increase the 13-methyl group in M would have to move in the direction of the cytoplasmic side of the membrane in order to keep the orientation of the transition dipole moment about constant.

The experiments show that the orientation of the transition dipole moment in M remains constant during its entire lifetime. Evidence was recently presented for an irreversible step between an early and late M intermediate [16]. This transition, which is believed to switch the chromophore from a state with Schiff base accessible to the extracellular medium to one with access to the cytoplasm, is accompanied by large enthalpy and entropy changes [17], suggesting a major conformational change [17,18]. Our results show that the chromophore orientation is not affected by these events.

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## REFERENCES

[1] Mathies, R.A., Lin, S.W., Ames, J.B. and Pollard, W.T. (1991) Ann. Rev. Biophys. 20, 491–518.
[2] Heyn, M.P., Cherry, R.J. and Müller, U. (1977) J. Mol. Biol. 117, 607–620.
[3] Dér, A., Száraz, S. and Czégé, J. (1988) Biophys. J. 54, 1175–1178.
[4] Breton, J. and Nabedryk, E. (1989) Biochim. Biophys. Acta 973, 13–18.
[5] Van Gurp, M., van Ginkel, G. and Levine, Y.K. (1989) J. Theor. Biol. 131, 333–342.
[6] Lewis, B.A., Rosenblatt, C., Griffin, R.G., Courtemanche, J. and Herzfeld, J. (1985) Biophys. J. 47, 143–150.

[7] Otto, H., Marti, T., Holz, M., Mogi, T., Lindau, M., Khorana, H.G. and Heyn, M.P. (1989) Proc. Natl. Acad. Sci. USA 86, 9228–9232.
[8] Heyn, M.P. (1979) FEBS Lett. 108, 359–364.
[9] Clark, N.A., Rothschild, K.J., Luippold, D.A. and Simon, B.A. (1980) Biophys. J. 31, 65–96.
[10] Michel-Villaz, M., Roche, C. and Chabre, M. (1982) Biophys. J. 37, 603–616.
[11] Seiff, F., Westerhausen, J., Wallat, I. and Heyn, M.P. (1986) Proc. Natl. Acad. Sci. USA 83, 7746–7750.
[12] Heyn, M.P., Westerhausen, J., Wallat, I. and Seiff, F. (1988) Proc. Natl. Acad. Sci. USA 85, 2146–2150.
[13] Hauß, T., Grzesiek, S., Otto, H., Westerhausen, J. and Heyn, M.P. (1990) Biochemistry 29, 4904–4913.
[14] Earnest, T.N., Roepe, P., Braiman, M.S., Gillespie, J. and Rothschild, K.J. (1986) Biochemistry 25, 7793–7798.
[15] Lin, S.W. and Mathies, R.A. (1989) Biophys. J. 56, 653–660.
[16] Váró, G. and Lanyi, J.K. (1991) Biochemistry 30, 5008–5015.
[17] Váró, G. and Lanyi, J.K. (1991) Biochemistry 30, 5016–5022.
[18] Ormos, P. (1991) Proc. Natl. Acad. Sci. USA 88, 473–477.