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Electric field induced conformational changes of bacteriorhodopsin in purple membrane films

I. DC field effects

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Abstract. Electric field-induced absorption changes of bacteriorhodopsin were studied with different samples of purple membranes which were prepared as randomly oriented and electrically oriented films of purple as well as cation-depleted blue bacteriorhodopsin. The absorption changes were proportional to the square of the field strength up to ≈ 300 kV/cm. The electric field from the intracellular side to the extracellular side of the purple bacteriorhodopsin induces a spectrum change, resulting in a spectrum similar to that of the cation-depleted blue bacteriorhodopsin. When the field was removed, the purple state was regenerated. The blue state was mainly affected by an electric field in the opposite direction, suggesting a reversible interaction with the Schiff's base bond of the retinal. Since the field-induced reaction of bacteriorhodopsin was observed in the presence of a concomitant steady ion flux, it is assumed that the generation of a local diffusion potential may play an important role in these spectral reactions. Although the fragments were fixed in the dried film, electric dichroism was observed. The dichroic contribution of the total absorbance change was about 15%. The angular displacement of the retinal transition moment was calculated to be 1.5° toward the membrane normal.

Key words: Bacteriorhodopsin, blue membrane, purple membrane films, electric field-induced states, electric dichroism

Introduction

Electrooptical measurements of bacteriorhodopsin in purple membranes revealed that conformational transitions of bacteriorhodopsin molecules are induced by the electric field pulses both in suspensions (Shinar et al. 1977; Hess 1978; Tsuji and Neumann 1981a, b, 1983) and in dried films (Borisevitch et al. 1979; Lukashev et al. 1980; Chamorovsky 1983).

The absorbance change induced by an electric field of 2–20 kV/cm with a duration < 100 µs in purple membrane suspensions consists of anisotropic contributions (the dichroism) and isotropic contributions (the chemical changes). It has been suggested that these reactions correspond to changes in the orientation of both retinal and tyrosine and/or tryptophan residues. In addition, the reactions of the microenvironment of aromatic amino acid residues concomitant with pK shifts in at least two types of proton binding site have been discussed (Tsuji and Neumann 1983). Since the electric field induced pH changes are opposite to the sequence of pH increase and decrease of purple membrane suspensions following a laser pulse, the electric field-induced conformational change may be considered as an intramolecular feedback on proton binding and release (Tsuji and Neumann 1981b). The mechanism of field response is explained with saturated induced dipole moments which may be caused by an increase of the distance of ion pairs in bacteriorhodopsin in a cooperative manner (Tsuji and Neumann 1983).

An effect of the electric field on the overall orientation of purple membrane fragments has been observed when an exponentially decaying electric field (Shinar et al. 1977; Tsuji and Rosenheck 1979) or a relatively low, long-lasting electric field (100 V/cm, 1–2 min) (Keszthelyi 1980; Kimura et al. 1981; Todorov et al. 1982; Kimura et al. 1984; Kahn and Shu-Itu 1984; Stoylov et al. 1984) was used.

A spectroscopic method using dried films of purple membrane has been applied to reduce the light scattering effect (Korenstein and Hess 1977a, b) and also to avoid the overall fragment orientation in the electric field. We used this method and studied

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in detail the electric field effects on bacteriorhodopsin with randomly and electrically oriented membrane films of purple and blue bacteriorhodopsin, the latter being cation-depleted (Kohl et al. 1984; Kimura et al. 1984b; Chang et al. 1985). A theory for the electric dichroism of the system where the fragments are fixed was developed.

Experimental

Materials

Purple membranes were isolated from *Halobacterium halobium* S9 strain according to Oesterhelt and Stoeckenius (1974). Purple membranes were suspended in double distilled water ($OD_{570} \approx 12$, pH ≈ 6). For preparation of blue species by electric dialysis, a purple membrane suspension was placed into a dialysis membrane tube. The tube was hung between two platinum electrodes separated by 3 cm and immersed in water. A voltage of 50 V was applied for 3 h, in this time the membranes changed their colour from purple to blue with a yield of $\approx 70\%$. The percentage was estimated on the basis of $\varepsilon_{565} = 63{,}000$ (Oesterhelt and Hess (1973) and $\varepsilon_{603} = 52{,}000$ (Kohl et al. 1984). The blue colour was stable, as long as the sample was kept cation-depleted.

In order to prepare a semi-transparent electrode, tantal and then gold were spattered on an optical glass plate in vacuum (thickness 25 nm). Tantal stabilizes the gold layer on the glass plate. For randomly oriented samples the purple membrane suspension in the purple (pH ≈ 6) or blue state (pH ≈ 4.5) was spread on the electrode and dried at 50% room humidity. The electrophoretically oriented samples were prepared according to Váró (1981). Two semi-transparent gold electrodes were used as cathode and anode, respectively. The purple membrane suspension was in contact with both electrodes, which were separated by ≈ 1 mm. When 4–5 V was applied for 5–15 s, the purple membrane sheets migrated and were attached with the intracellular side facing the anode (see Fig. 1a), concomitant with a colour change from purple to blue. The extent of the change depends on the magnitude of the applied voltage and its duration. Then, excess water was removed from the side with a small syringe, and the layers were equilibrated at 50% humidity.

Another gold layer was spattered both on the random and on the oriented purple membrane films. The amount of the purple state relative to the total number of bacteriorhodopsin molecules was determined spectrophotometrically. The absorption peak of the natural purple membrane films (100% purple) shifted to 550 nm, probably because of an overestimation of the absorption of tantal and gold layers; the absorption minimum of the (Ta + Au) electrode is 550 nm. The thickness of the purple membrane films was 1–2 μm as measured from scanning electron microscope photographs (Korenstein and Hess 1982). The orientation of the membrane fragments was analyzed in an electron microscope using the Pt-C shadowing technique (Fisher et al. 1978).

Electro-optical measurements

The arrangement for the measurement of the electric dichroism is shown schematically in Fig. 2. Light from a 400 W halogen lamp passed through a monochromator (Bausch & Lomb) and a Glan-Thompson polarizer, and reached the purple membrane film through a gold electrode. A sample holder was set at the angle φ ($\varphi = 0 - \pi/3$) with respect to the direction of the light beam.

For the electric pulses a DC power supply NT 05 (P-T-M electronic) was used, allowing a voltage of up to 50 V, which corresponds to an electric field, $E = 250$ kV/cm for a 2 μm film. The intensity of the transmitted light was detected by a photomultiplier (EMI 9634 QR) and amplified. The output of the amplifier was displayed on an oscilloscope and was simultaneously recorded by a pen recorder.

Field induced absorbance changes measured with various intensities of the monitoring light from 0.2 mW/m^2 to 13 mW/m^2 showed only a slight dependence at $\lambda = 555$ nm; the signal with a light intensity of 13 mW/m^2 was 0.5% larger than that with 0.2 mW/m^2. At $\lambda = 646$ nm no dependence was detected. Therefore, in this light intensity range the effect of the monitoring light can be neglected.

Electric current measurements

The electric current which passes across the purple membrane film was measured with a 153 Microvoltammeter (Keithley Instruments) simultaneously with the optical signal. The resistance of the films was calculated from the stable current through the samples when 50 V was applied in the field direction, I, defined as the direction of the orienting field (from the first or lower electrode to the second or upper electrode (see Fig. 1b)).

Theoretical background for the electric field induced absorbance change of the purple membrane dried film

In general, the electric field induced absorbance change, ΔA_σ, consists of a chemical part, ΔA^{ch}, and a

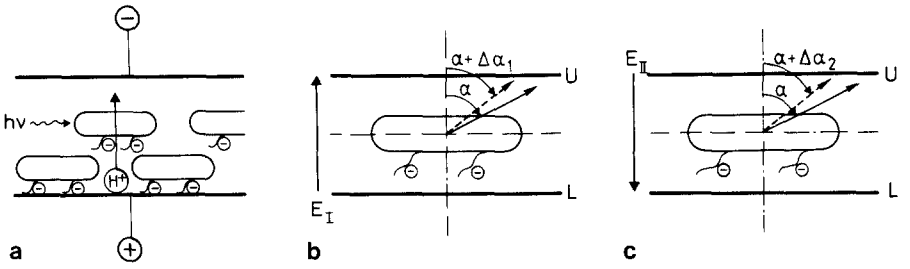

Fig. 1. a Geometry of the electrophoretically oriented purple membranes. Purple membranes migrate to the anode and have the intracellular side facing towards the anode. The arrow shows the direction of the light driven proton pumping. **b** and **c** Geometrical relations between electrophoretically oriented purple membranes and applied electric field directions E_I and E_{II}; *L*, the lower (first) electrode; *U*, the upper (second) electrode. Arrows show the direction of the retinal transition moment in the absence of the electric field (→) and in the presence of the electric field (⇢)

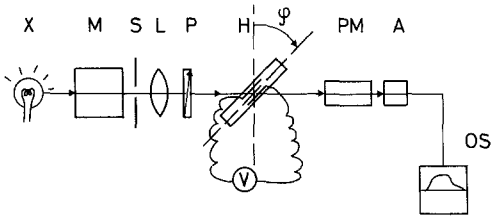

Fig. 2. Schematic diagramm of the set-up for measurements of electric dichroism of purple membrane dried films. *X*, halogen lamp; *M*, monochromator; *S*, slit; *L*, lens; *P*, polarizer; *H*, sample holder; *PM*, photomultiplier; *A*, amplifier; *OS*, oscilloscope

linear dichroism part, $\Delta A_\sigma^{\text{rot}}$,

$$\Delta A_\sigma = \Delta A^{\text{ch}} + \Delta A_\sigma^{\text{rot}} \tag{1}$$

where σ is the light polarization direction with respect to the electric field (Tsuji and Neumann 1981 a).

The chemical change

Assuming that ΔA_1^{ch} and ΔA_2^{ch} are the chemical terms of the absorbance change when the electric field is applied from the intracellular side to the extracellular side (subscript 1) and vice versa (subscript 2), and a is the molar fraction of bacteriorhodopsin molecules in which the intracellular side faces towards the first electrode, the total chemical contribution of the absorbance change is given by

$$\Delta A^{\text{ch}}(\text{I}) = a\,\Delta A_1^{\text{ch}} + (1-a)\,\Delta A_2^{\text{ch}} \tag{2-a}$$

for the field direction I, and

$$\Delta A^{\text{ch}}(\text{II}) = (1-a)\,\Delta A_1^{\text{ch}} + a\,\Delta A_2^{\text{ch}} \tag{2-b}$$

for the opposite field direction, II (see Fig. 1 b and c).

The electric dichroism

Since the purple membrane fragments are fixed on the electrode, the electric dichroism in such a case is different from the system in which particles are freely rotating (Fredericq and Houssier 1973). When the sample holder is set at $\varphi = \pi/4$, and the orientation is 100% ($a = 1$), the reduced dichroism for the field direction I is given by

$$\frac{\Delta A^{\text{rot}}(\text{I})}{A} = \frac{3}{2}\sin\alpha\cos\alpha\sin\Delta\alpha_1\cos\Delta\alpha_1 \tag{3-a}$$

and for the field direction II by

$$\frac{\Delta A^{\text{rot}}(\text{II})}{A} = \frac{3}{2}\sin\alpha\cos\alpha\sin\Delta\alpha_2\cos\Delta\alpha_2, \tag{3-b}$$

where A is the absorbance for $E = 0$, α is the angle of the retinal transition moment at $E = 0$ with respect to the membrane normal, $\Delta\alpha_1$ and $\Delta\alpha_2$ are the altered angles to the membrane surface due to fields I and II, respectively. Detailed derivations are given in the Appendix.

Results

Six types of membrane films of bacteriorhodopsin were prepared in order to study the effects of its orientation as well as its purple and blue state with respect to the electrooptical property of bacteriorhodopsin, as shown in Table 1. Purple membranes in samples A-1 and A-2 were randomly oriented, that is, about 50% of purple membranes had their intracellular side facing the lower electrode and the other 50% had their extracellular side facing the lower electrode. Sample A-1 was made from the purple membrane and sample A-2 from electrodialyzed blue membrane. In the electrophoretically oriented samples nearly all purple membranes have the intracellular side facing the lower electrode (see Fig. 1 a). The ratio of the purple state to the total amount of bacteriorhodopsin in the films decreased from B-1 to B-4. The absorption maximum, the amount of the purple state as a percentage of the total bacteriorhodopsin, and the thickness of the

Table 1. Characterization of the purple membrane films

Sample no.	Purple membrane	Geometry	Absorption max/nm	% of the purple state	Thickness/ μm	Resistance in DC field I/ Ω · m
A-1	Natural	Random	550	100	1.5	10^8
A-2	Electrodialyzed	Random	585	30	2.0	10^{10}
B-1	Natural	Oriented by $E = 40$ V/cm, 5 s	550	100	1.5	10^9
B-2	Natural	Oriented by $E = 50$ V/cm, 5 s	565	70	2.2	10^9
B-3	Natural	Oriented by $E = 50$ V/cm, 10 s	585	30	1.2	10^9
B-4	Natural	Oriented by $E = 50$ V/cm, 15 s	600	0	1.2	10^{10}

Table 2. Relaxation times of the electric field induced absorption change

1. Due to field I (50 V) at the negative peak

Sample	λ/nm	τ_{on}/s (partial amplitude)		τ_{off}/s (partial amplitude)		
A-1	555	12 (75%)	76 (25%)	2.4 (50%)	13 (50%)	
A-2	555	10 (− 1%)	58 (101%)	4.7 (27%)	20 (73%)	
B-1	555	4.9 (− 10%)	37 (110%)	1.2 (23%)	28 (67%)	
B-2	555	3.6 (− 8%)	87 (108%)	9.9 (59%)	58 (41%)	
B-3	597	67 (100%)		3.9 (32%)	32 (68%)	
B-4	621	< 1 (36%)	78 (64%)	< 1 (59%)	7.3 (20%)	87 (21%)

2. Due to field II (50 V) at the negative peak

Sample	λ/nm	τ_{on}/s (partial amplitude)		τ_{off}/s (partial amplitude)	
B-1	555	11 (18%)	54 (82%)	4.0 (42%)	33 (58%)
B-2	555	12 (16%)	73 (84%)	5.5 (47%)	68 (53%)
B-3	597	4.8 (32%)	79 (68%)	5.6 (50%)	72 (50%)
B-4	621	6.3 (37%)	66 (63%)	4.0 (46%)	41 (54%)

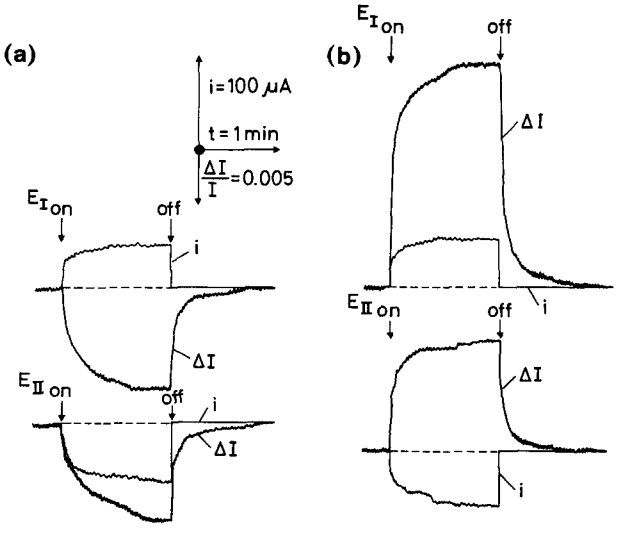

Fig. 3. Intensity changes of transmitted light (——) and the electric current (——) of the purple membrane dried film B-1 due to electric fields I and II. $E = 330$ kV/cm; $\varphi = 0$. **(a)** $\lambda = 555$ nm, **(b)** $\lambda = 646$ nm

film, as well as the resistance in field I for the six samples are listed in Table 1. Note that the resistance of the samples with higher content of the cation-depleted blue state was higher than that of purple samples, which might be due to the low ion content of the blue membranes samples (see discussion).

Figure 3 shows typical optical signals at wavelengths 555 and 646 nm and corresponding electric currents due to DC electric fields. The amplitude of the absorbance change was linearly proportional to the square of the field strength E^2, up to $E \approx 300$ kV/cm, as shown in Fig. 4. Since ΔA is proportional to E^2 with the thickness being d

$$\Delta A \propto E^2 d = V^2/d\,, \tag{4}$$

where V is the applied voltage. Therefore, amplitudes were normalized by the inverse of the thickness of the film, so that all samples can be compared for the same electric field strength and for the same

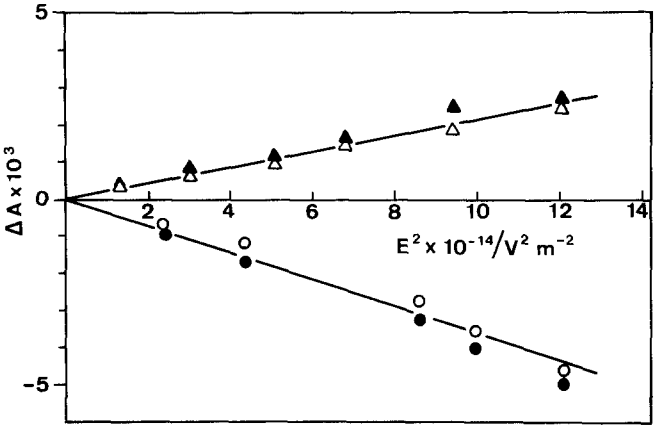

Fig. 4. Field strength dependence of the absorbance change of the purple membrane dried film A-1. ○ due to E_I at 555 nm; ● due to E_{II} at 555 nm; △ due to E_I at 646 nm; ▲ due to E_{II} at 646 nm.

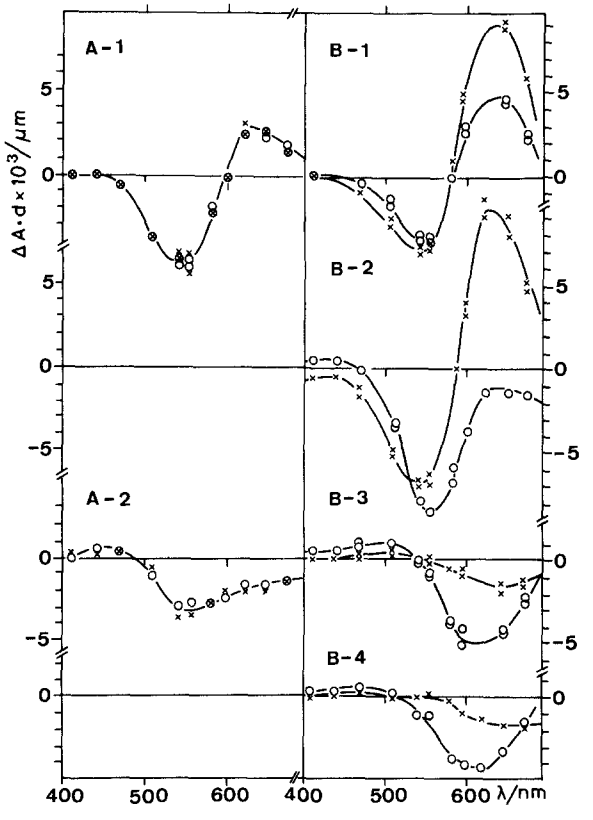

Fig. 5 Wavelength dependence of the electric field-induced absorbance change for the six samples, normalized by the inverse of the thickness of films (see text). –x– due to DC field I; –○– due to DC field II

thickness. Such normalized difference spectra due to fields I and II are shown in Fig. 5.

As a result of the fields I and II, the purple sample A-1 shows a negative peak at 555 nm and a positive peak at 620 nm while the blue sample, A-2, shows a small positive peak at 430 nm and a broad negative peak at 555 nm.

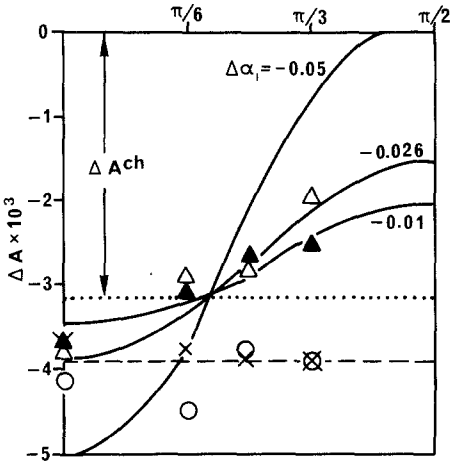

Fig. 6. Electric dichroism of the purple membrane dried film B-1. —— theoretical curves for the horizontal component of absorbance change with various values of $\Delta\alpha_1$; --- theoretical curve for the vertical component of absorbance change; the horizontal component of absorbance change due to the field I (▲) and II (△) and the vertical component of absorbance change due to the field I (×) and II (○)

For the electrically oriented samples, the effect of field I was different from that of field II. In the field I, which corresponds to the field direction from the intracellular side to the extracellular side, the absorbance decreased at 555 nm and increased at 640 nm for the purple sample, B-1. This trend was more marked for the sample B-2. For the blue samples, B-3 and B-4, a small absorbance increase around 450 nm and a decrease around 670 nm were observed. In the field II, the positive peak at 640 nm decreased and the negative peak at 555 nm seemed to shift to the longer wavelength ($\approx$ 620 nm), on going from B-1 to B-4.

Further electrooptical investigation with an AC field suggests the existence of, at least, two more steps at the beginning of the reaction sequence in the electric field (Tsuji and Hess, in preparation). Note also that difference spectra for B-2 or B-3 cannot be obtained by superposition of those for B-1 and B-4, suggesting that there is, at least, one intermediate state between the pure purple and the pure blue state.

Both the field-on and field-off processes of the optical changes due to fields I and II could be analyzed by a sum of two exponentials (Table 2). In general, the field-off processes were slightly faster than the field-on processes. Except for B-2, both field-on and -off processes due to field I were faster than those due to field II. The current changes consisted of a very fast phase of the order of μs and a subsequent slow phase of the order of *s*.

The electric dichroism of purple sample B-1 was measured in the DC electric fields (both I and II). Figure 6 shows the electric dichroism for the sample

B-1 at $\lambda = 555$ nm and $E = 330$ kV/cm and the theoretical curves of Eq. (10-a) (see Appendix) for $a = 1$ as well as including the chemical contribution. Since the dichroic signals in both field directions are similar, $\Delta\alpha_1$ and $\Delta\alpha_2$ should be almost the same (see Eqs. (3-a) and (3-b)). The rotational contribution for $\varphi = 0$ (the vertical polarization) to the total absorbance change is 15%. According to Eq. (3-a), $\Delta\alpha_1$ is calculated to be $-1.5°$, if α is assumed to be 70° (Heyn et al. 1977; Korenstein and Hess 1978). This means that the DC electric field alters the retinal transition moment by 1.5° toward the membrane normal. It should be noted, that at 646 nm no dichroism was observed.

Discussion

Our experiments show that the conformational change in the purple and blue states of bacteriorhodopsin due to a DC field is proportional to the square of the field strength, as expected for the case of the induced dipole equilibria which is not saturated in the range of the applied electric field. In addition we found that the field-induced reaction is dependent on the direction of the applied field.

Since the purple membrane is electrically and structurally asymmetric, the effect due to the field from the intracellular side to the extracellular side is expected to be different from that in the opposite field direction. In fact, for the electrically oriented samples B-1, 2, 3 and 4 the absorbance changes both in the amplitude and kinetics are different for the two field directions. For the samples A-1 and 2, which were dried under the condition of random orientation, the signals in response to fields I and II are practically identical both in amplitude and kinetics over the whole wavelength range, indicating that the populations of the two oppositely oriented purple membrane fragments are equal ($a \approx 1/2$, see Eqs. (2-a) and (2-b)).

The observation of an electric current is of special interest, since it suggests that a steady ion flow may be a necessary condition for the field induced conformational change of bacteriorhodopsin. The site dependency of the spectroscopic reaction concomitant with ion flows suggests the following sequence of events. The DC electric field generates an overall ion current through the bulk of many bacteriorhodopsin layers. From independent observations in this laboratory it is expected that the bulk of bound ions on the surface of each purple membrane layer – either sodium ions, potassium ions, protons or other ions – may readily maintain such a current. This overall current of movable ions through the membranes generates a local diffusion potential across each single purple membrane, which builds up a local electrical field strength in the order of 100 kV/cm across the individual membranes with a thickness of roughly 50 Å. Because the overall field effect is direction-dependent, the local configuration within bacteriorhodopsin which responds to the local field, must be located in a site-specific manner. A possible candidate for this configuration is the recently discovered binding site for metal ions in bacteriorhodopsin. This site binds cations stoichiometrically as an obligatory component for the purple state of the pigment and the function of the proton pump (Kohl et al. 1984). This cation binding site might well be considered as an intrinsic field sensor. By analogy, we expect that such cation binding senses an electric field strength of the order of 10 kV/cm on the bases of earlier electrical field relaxation studies (Eigen and De Maeyer 1963).

This interpretation is in accord with a direction-dependent generation of an absorption spectrum similar to that of the blue membrane obtained upon dissociation of the cation (Kohl et al. 1984). Because of the location of the cation at only one site in the membrane, a release of the ion in the opposite direction is obviously hindered. Also, in other experiments, we observed that the cation release process occurs in two steps. This observation fits with the results reported here that the field-induced absorption shift is at least a two-step transition. Note that the blue state of the sample B-1, which appears in electric field I, is different from the cation-depleted stable blue state, because the former returns to the purple state when the field is removed. This means that the electric field-induced cation release in sample B-1 is a reversible change.

On the other hand, blue membranes are depleted of cations. The purple state cannot be regenerated by the opposite field direction, II. The difference spectrum of sample B-4 in response to field II indicates a disappearance of the blue state without the appearance of a spectrophotometrically visible product, at least in the range between 400 and 700 nm. This suggests that the unstable Schiff's base bond of retinal is reversibly broken. Retinal, bound in the cation-depleted blue state, is known to be unstable against light illumination, and the field response observed here might reflect the same mechanism (Engelhard, private communication).

Until now it has not been clear whether the electric field promotes or blocks the photocycle of bacteriorhodopsin in dried film (Borisevitch et al. 1979; Lukachev et al. 1980; Chamorovsky et al. 1983). The problem is currently under investigation, especially with respect to the interpretation given here.

Measurements of the electric dichroism gives us more information about the conformational change of bacteriorhodopsin. Observation of the electric

dichroism at 555 nm in the purple sample indicates that the conformational change involves not only the above mentioned isotropic change of the state from purple to blue but also an anisotropic change in the retinal transition moment. As is described in the results section, the ratio of the rotational contribution to the total absorbance change is 15% (see Eq. (1)) and the angular displacement of the retinal transition moment is 1.5° toward the membrane normal at $E = 330$ kV/cm of both directions I and II. The same degree and sign for the dichroism due to fields I and II suggests that the electric dipole moment along the retinal does not directly interact with the applied electric fields, but that the conformational change of the protein part – interacting with the cation – may dominate the direction of retinal.

The value of the angular displacement is rather small compared to that in suspensions, probably because the conformational change in the dried film is far below saturation. This is indicated by the square field dependence of ΔA, as well as by the rather slow kinetics of build-up compared to the field-off decay. On the other hand, the saturation of ΔA in suspensions starts at ≈ 5 kV/cm (Tsuji and Neumann, 1981a). This difference in sensitivity toward electric field perturbation may be due to the different state of hydration in both samples. Earlier it has been found that the kinetics of the photocycle of bacteriorhodopsin strongly depend on the state of hydration (Korenstein and Hess 1977b). In addition, we would expect that the mobility of ions would depend on the hydration condition. Therefore we suggest that the electric field strength required for saturation of the conformational change is dependent on the hydration state itself.

Appendix

Calculation of the electric dichroism for a fixed system such as dried films of purple membrane

The retinal chromophore transition moment vector, $\boldsymbol{\mu}$, of a bacteriorhodopsin molecule is expressed in the Cartesian coordinate system, $O-\xi\,\eta\,\zeta$, which is fixed on the purple membrane by

$$\begin{aligned}\mu_\xi &= \mu \sin\alpha \cos\beta\\ \mu_\eta &= \mu \sin\alpha \sin\beta\\ \mu_\zeta &= \mu \cos\alpha\,,\end{aligned} \tag{5}$$

where α is the angle between the membrane normal (ζ-axis) and $\boldsymbol{\mu}$ (see the inset of Fig. 7). $\boldsymbol{\mu}$ can be also expressed in the coordinate system $O-x\,y\,z$ which is fixed in the laboratory frame; the y-axis is the direction of the light beam and the x-axis and the z-axis are the horizontal and vertical directions of the

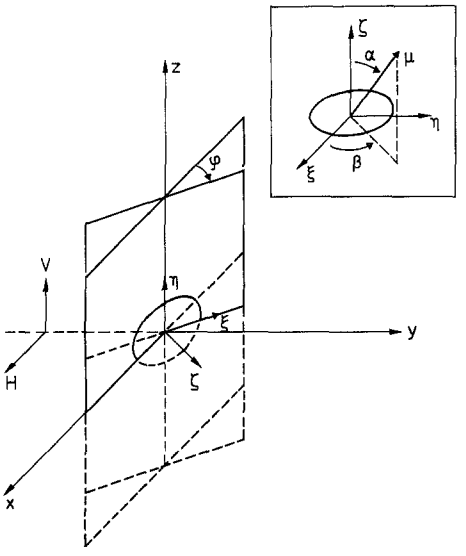

Fig. 7. A purple membrane fragment in Cartesian coordinate systems $O-\xi\,\eta\,\zeta$ (fixed on the fragment) and $O-x\,y\,z$ (fixed in the laboratory). ζ is the direction of the membrane normal, y is the direction of the light beam and x and z are vertical and horizontal directions of the polarizer, respectively. $\boldsymbol{\mu}$ is the transition moment of retinal

light polarization, respectively, as shown in Fig. 7. When the angle between the purple membrane plane (the $\xi-\eta$ plane) and the $x-z$ plane is φ, the x, y and z components of $\boldsymbol{\mu}$ are

$$\begin{aligned}\mu_x &= \cos\varphi\cdot\mu_\xi + \sin\varphi\cdot\mu_\zeta\\ \mu_y &= -\cos\varphi\cdot\mu_\zeta\\ \mu_z &= \mu_\eta\,.\end{aligned} \tag{6}$$

Then the absorbances with the horizontally and vertically polarized light are given by

$$\begin{aligned}A_x &= \varkappa\mu_x^2 = \varkappa\mu^2(\cos\varphi\sin\alpha\cos\beta + \sin\varphi\cos\alpha)^2\\ A_z &= \varkappa\mu_z^2 = \varkappa\mu^2\sin^2\alpha\sin^2\beta\,,\end{aligned} \tag{7}$$

where $\varkappa$ is a constant. Since the direction β is at random in the purple membrane film, the average for β results in

$$\begin{aligned}\overline{A_x} &= \frac{1}{2\pi}\int_0^{2\pi} A_x\,d\beta = \varkappa\mu^2\left(\tfrac{1}{2}\cos^2\varphi\sin^2\alpha + \sin^2\varphi\cos^2\alpha\right)\\ \overline{A_z} &= \frac{1}{2\pi}\int_0^{2\pi} A_z\,d\beta = \tfrac{1}{2}\varkappa\mu^2\sin^2\alpha\,.\end{aligned} \tag{8}$$

If the angle α is changed to $\alpha+\Delta\alpha_1$ and $\alpha+\Delta\alpha_2$ due to the electric fields I and II, the direction of which are from the intracellular side to the extracellular side and vice versa, as shown in Fig. 1, the horizontal and the vertical absorbances are given by

$$\begin{aligned}\overline{A_x^I} = \varkappa\mu^2\,[&a\,\{\tfrac{1}{2}\cos^2\varphi\sin^2(\alpha+\Delta\alpha_1)\\ &+\sin^2\varphi\cos^2(\alpha+\Delta\alpha_1)\}\\ &+(1-a)\,\{\tfrac{1}{2}\cos^2\varphi\sin^2(\alpha+\Delta\alpha_2)\\ &+\sin^2\varphi\cos^2(\alpha+\Delta\alpha_2)\}]\end{aligned} \tag{9-a}$$

$$\overline{A_z^I} = \tfrac{1}{2}\varkappa\mu^2\,\{a\sin^2(\alpha+\Delta\alpha_1) + (1-a)\sin^2(\alpha+\Delta\alpha_2)\}$$

for the electric field direction I, and

$$\overline{A_x^{II}} = \varkappa\mu^2[(1-a)\{\tfrac{1}{2}\cos^2\varphi\sin^2(\alpha+\Delta\alpha_1) + \sin^2\varphi\cos^2(\alpha+\Delta\alpha_1)\} + a\{\tfrac{1}{2}\cos^2\varphi\sin^2(\alpha+\Delta\alpha_2)+\sin^2\varphi\cos^2(\alpha+\Delta\alpha_2)\}]$$
$$\overline{A_z^{II}} = \tfrac{1}{2}\varkappa\mu^2\{(1-a)\sin^2(\alpha+\Delta\alpha_1) + a\sin^2(\alpha+\Delta\alpha_2)\} \quad (9\text{-b})$$

for the opposite field direction, II. When $\Delta\alpha_1$, $\Delta\alpha_2 \ll 1$, the absorbance changes are approximated as

$$\Delta A_x^{I} = \overline{A_x^{I}} - \overline{A_x} \approx \varkappa\mu^2(3\cos^2\varphi - 2)\sin\alpha\cos\alpha\{a\sin\Delta\alpha_1\cos\Delta\alpha_1 + (1-a)\sin\Delta\alpha_2\cos\Delta\alpha_2\}$$
$$\Delta A_z^{I} \approx \varkappa\mu^2\sin\alpha\cos\alpha\{a\sin\Delta\alpha_1\cos\Delta\alpha_1 + (1-a)\sin\Delta\alpha_2\cos\Delta\alpha_2\} \quad (10\text{-a})$$

for the field direction I and

$$\Delta A_x^{II} \approx \varkappa\mu^2(3\cos^2\varphi - 2)\sin\alpha\cos\alpha \cdot\{(1-a)\sin\Delta\alpha_1\cos\Delta\alpha_1 + a\sin\Delta\alpha_2\cos\Delta\alpha_2\}$$
$$A_z^{II} \approx \varkappa\mu^2\sin\alpha\cos\alpha\{(1-a)\sin\Delta\alpha_1\cos\Delta\alpha_1 + a\sin\Delta\alpha_2\cos\Delta\alpha_2\} \quad (10\text{-b})$$

for the field direction II. Note that ΔA_z is independent of φ. When $\varphi = 0$, $\Delta A_x = \Delta A_z$.

When $\varphi = \pi/4$ and $a = 1$, the reduced dichroism for the field I is calculated as

$$\frac{\Delta A^{rot}}{A} = \frac{\Delta A_z^{I} - \Delta A_x^{I}}{A} = \tfrac{3}{2}\sin\alpha\cos\alpha\sin\Delta\alpha_1\cos\Delta\alpha_1. \quad (11)$$

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