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Electric field induced conformational changes of bacteriorhodopsin in purple membrane films

II. Alternating field effects

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Abstract. Electric field induced conformational changes of bacteriorhodopsin were studied in six types of dried film (randomly and electrically oriented membranes of purple as well as cation-depleted blue bacteriorhodopsin) by measuring the frequency dependence of the optical absorbance change and the dielectric dispersion and absorption. For the purple bacteriorhodopsin the optical absorbance change induced by alternating rectangular electric fields of ±300 kV/cm altered the sign twice in the frequency range from 0.001 Hz to 100 kHz (around 0.03 Hz and 100 kHz), indicating that the electric field induced conformational change in these samples consists of, at least, three steps. Similarly, it was found for the blue bacteriorhodopsin that at least two steps are involved. In accord with optical measurements, the dielectric behaviour due to alternating sinusoidal electric fields of ±6 kV/cm in the frequency range from 10 Hz to 10 MHz showed two broad dispersion/absorption regions, one below 1 kHz and the other around 10–100 kHz. This suggests that the conformational change of bacteriorhodopsin is also reflected by its dielectrical properties and that it is partially induced at 6 kV/cm. Including previous results obtained by analysis of the action of DC fields on purple membrane films, a model for a field-induced cyclic reaction for purple as well as blue bacteriorhodopsin is proposed. In addition it was found that there are electrical interactions among purple membrane fragments in dried films.

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

Introduction

It is known that an externally applied electric field induces conformational changes of bacteriorhodopsin 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 et al. 1983; Maximychev et al. 1984; Tsuji and Hess 1986).

In a previous paper (Part I of this series, Tsuji and Hess 1986) we have shown the action of DC fields with a duration of several minutes on purple bacteriorhodopsin and cation-depleted blue bacteriorhodopsin, prepared as randomly oriented and electrically oriented films. An electric field directed from the intracellular side to the extracellular side of purple membrane induces a change in the absorption spectrum of the purple bacteriorhodopsin similar to that of the purple-blue transition, while no significant change is observed in the case of blue bacteriorhodopsin. An electric field with the opposite direction mainly interacts with the retinal of blue bacteriorhodopsin. The reactions are reversible. From the electric dichroism of purple bacteriorhodopsin the angular displacement of the retinal transition moment is estimated to be 1.5° towards the membrane normal. Thus, the measurements with DC electric fields yield information about the conformation of bacteriorhodopsin at the steady state in the electric field.[1]

In an extension of these studies we have investigated the action of alternating fields on bacteriorhodopsin in order to see whether there are fast reactions at the early stage of the conformational change. In this paper we report the changes observed in the optical absorbance due to alternating fields of rectangular shape in the frequency range from

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1 Discrepancies between our results in Part I and the results of Maximychev et al. (1984) can be explained by the different system of electrodes used. They used two different metals so that the electrode potential at one interface between the purple membrane film and the electrode is different from that at the other interface. In this case the system itself is a kind of a battery – indeed they observed a "dark" potential –, and therefore the results are not comparable.

0.001 Hz to 100 kHz. The field strength ranges from 100 kV/cm to 300 kV/cm, which is comparable to that used in the investigation with DC fields. In addition, measurements of dielectric dispersion and absorption were performed with alternating sinusoidal fields in the frequency range from 10 Hz to 10 MHz having the comparably low field strength of 6 kV/cm. The occurrence of dielectric relaxation processes gives additional information about the conformational change of bacteriorhodopsin of low electric field strength and/or at high frequencies.

Experimental

Materials

Purple membranes were isolated from *Halobacterium halobium* S 9 strain according to Oesterhelt and Stoeckenius (1974). The blue state of bacteriorhodopsin was prepared by electrodialysis (Tsuji and Hess 1986). The blue colour was stable, as long as the sample was kept cation-depleted.

The randomly and electrically oriented purple membrane films were prepared on a semi-transparent glass electrode spattered with tantal and gold (the lower electrode *L*, Fig. 1). Subsequently, gold was spattered on the film (upper electrode *U*, Fig. 1), as has been described previously (Tsuji and Hess 1986). For the optical measurements an area of 280 mm^2 was spattered while for the dielectric measurements samples with various areas from 4 mm^2 to 280 mm^2 were prepared. The orientation of the membrane fragments was checked by the Pt–C shadowing method for electron microscopy (Fischer et al. 1978). The thickness of the film was determined using scanning electron microscope photographs (Korenstein and Hess 1982). For the intermediate samples the shift from purple to blue (in percent) was estimated according to the wavelength of the absorption maximum.

Electrooptical method

The same set-up as described in Part I (Tsuji and Hess 1986) was used with exception of the DC power supply.

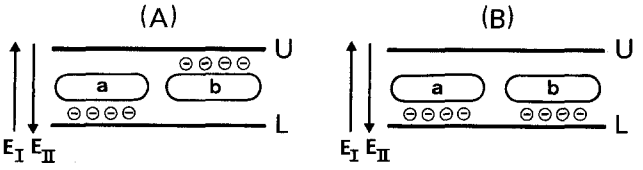

Fig. 1 A and B. Geometric relation between purple membrane fragments (*a* and *b*) and the direction of the externally applied electric fields (E_I and E_{II}) for the unoriented (**A**) and the oriented (**B**) samples. The field directions I and II are defined as the direction from the lower electrode (*L*) to the upper electrode (*U*) and vice versa, respectively. The intracellular side of the purple membrane is shown with negative charges

Rectangular pulses in the frequency range from 0.001 Hz to 100 kHz from a pulse generator (Model 505, Exact Electronics Inc.) were amplified by a factor of 100. Up to ± 100 V was available, corresponding to an electric field of ± 500 kV/cm for a film of 2 μm thickness. Each field inversion after amplification was faster than 1 μs. The duration of the alternating field was of the order of minutes. All experiments were carried out in $\approx 50\%$ room humidity at 20 °C.

The absorbance change ΔA due to the electric field E is calculated from the light intensity change ΔI by

$$\Delta A = -\log\left(1+\frac{\Delta I}{I_0}\right), \tag{1}$$

where I_0 is the light intensity in the absence of the field (Fredericq and Houssier 1973).

Dielectric method

The frequency dependence of the capacity C_x and the conductance G_x of the dried films (including the sample holder) was measured in the frequency range from 10 Hz to 10 MHz with two admittance bridges, Hewlett Packard 192 A and Boonton 33 D/1. The spattered sample film is considered as a plate condenser of capacitance $C = \varepsilon_0 \cdot \varepsilon(\nu)\, S/d$, where S is the surface area, d the distance between the two metal electrodes and ε_0 the vacuum permittivity. The dielectric properties of the bacteriorhodopsin films are described by the frequency dependent complex permittivity $\varepsilon(\nu) = \varepsilon'(\nu) - j\varepsilon''(\nu)$ with the dispersive real part $\varepsilon'(\nu)$ and the absorptive imaginary part $\varepsilon''(\nu)$, where ν is the frequency and j the imaginary unit. The latter term contains both dielectric losses and losses due to the specific DC conductivity. After correcting for the purely capacitive contribution C_0 of the cell holder one has $(C_x - C_0) \propto \varepsilon'$ and $G_x/2\pi\nu \propto \varepsilon''$ (Böttcher and Bordewijk 1978).

A thin polyester film (Mylar B 12, DuPont) with known thickness (12 μm) and dielectric constant ($\varepsilon' \approx 3.3$ at 1 kHz and 25 °C) spattered with the same metal films was used as reference sample.

Results

Six different types of purple membrane films as listed in Table 1, were used for the measurements. The samples A-1 and A-2 are the native purple and the electrodialyzed blue bacteriorhodopsin films, in which membrane fragments orient randomly. As illustrated in Fig. 1 A, the word "random" is used for an approximately equal distribution of the two possible orientations, one with the intracellular side of the membrane and the other with the extracellular side facing toward the lower electrode. Samples B-1 to B-4 are the

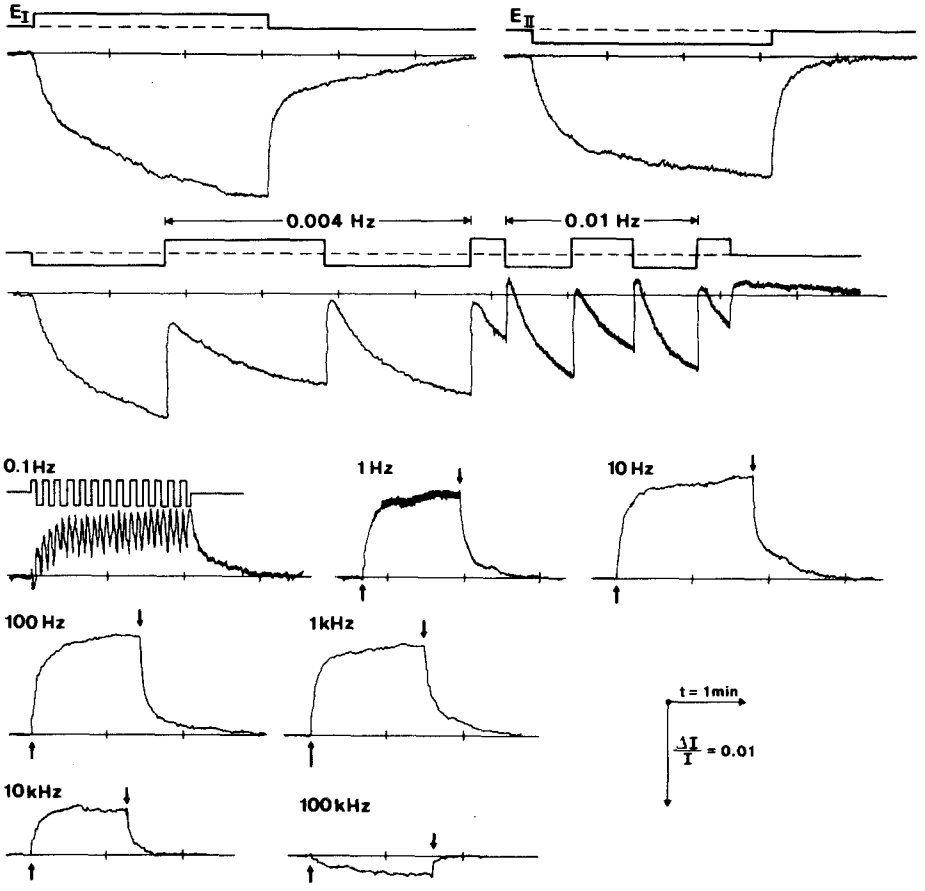

Fig. 2. Changes of transmitted light intensity ΔI of the purple membrane dried film A-1, due to the electric field DC and AC with frequency range from 0.004 Hz to 100 kHz; $\lambda = 555$ nm; $E = \pm 330$ kV/cm. Corresponding electric field directions are shown by rectangles above $\Delta I/I$ curves up to 0.1 Hz. Otherwise arrows show the time when the electric field is applied (↑) and removed (↓). The positive ΔI points downward

Table 1. Characterization of the purple membrane films

Sample no.	Purple membrane	Geometry	Absorption max./nm	% of the purple state
A-1	native	random	550	100
A-2	electro-dialyzed	random	585	30
B-1	native	oriented	550	100
B-2	native	oriented	565	70
B-3	native	oriented	585	30
B-4	native	oriented	600	0

electrically oriented samples, the intracellular side of the membrane facing toward the lower electrode (see Fig. 1 B). These are characterized by the distribution of spectral components – from 100% purple to 100% blue.

A. Electrooptical measurements

We use the definition for the electric field direction as follows: the electric field I (E_I) is directed from the lower electrode to the upper electrode (the direction from the intracellular side to the extracellular side of the purple membrane for the electrically oriented samples), and the direction of the electric field II (E_{II}) is opposite to that of E_I (see Fig. 1).

Figure 2 shows typical optical signals in terms of changes of transmitted light intensity ΔI for the sample A-1 caused by the alternating field with various frequencies. For comparison, the signal induced by DC fields are given in the first row of this figure. When the frequency is less than 0.003 Hz, the signals reach the same steady states as those due to DC fields I and II, that is, the same conformational changes as those caused by DC fields are induced.

As is seen in the second row of Fig. 2, at the frequency of 0.004 Hz such steady states are not observed anymore because the direction of the electric field is changed before the steady state is established. The observed signal is, then, a superposition of the decaying process of the former field effect and the build-up process of the currently acting field; for example, a sum of the decaying process due to E_I and the rise-up process due to E_{II}. When the frequency is 0.01 Hz, the signal crosses the base line shortly after each field inversion. This is an indication of the existence of a fast process, which causes an absorbance change with the sign opposite to that of the DC signal, that is, the conformation of bacteriorhodopsin induced by this process is different from that induced by DC fields.

At 0.1 Hz, only during the first half of the first period does ΔI increase (note that positive ΔI points downward in Fig. 2), crossing the baseline after the first field inversion. Subsequently, the transient peaks of ΔI after each field inversion decreases gradually, and after a few inversions ΔI changes periodically around a negative steady state. During 5 s the amplitude of the slow process (DC signal) is so small that the contribution of the fast process to the conformation of bacteriorhodopsin is accumulated.

At 1 Hz the positive peak at the beginning is not resolved anymore, and the amplitude of the negative steady state is larger than that at 0.1 Hz, reflecting the fact that the contribution of the slow process diminishes further. The amplitude reaches a maximum around 10 Hz and then decreases with increasing frequency in the range from 10 Hz to 10 kHz. This suggests that this fast process can not completely follow the polarity change above 100 Hz. The time course of the absorbance change at 10 Hz can be analysed by an exponential function with a time constant of ≈ 5 s. This may correspond to the first exponential component in DC signals (see Table 2 in Part I).

At 100 kHz the sign of the signal is reversed again, indicating that there exists a still faster process which causes a signal with the same direction as in the case of the DC signal. Still higher frequencies would be necessary in order to characterize the absorbance change associated with this process in terms of time constant and amplitude. Since no faster exponential component was found at the early stage of the rise-up curve at 10 Hz, the time constant should be at least one order of magnitude lower than 5 s.

The frequency dependence of the steady state of the absorbance change for the six samples is shown in Fig. 3. Since ΔA for both randomly and electrically oriented samples is proportional to the square of the field strength, E^2, up to $E = \pm 350$ kV/cm as shown in Fig. 4, the amplitude is normalized by the inverse of the film thickness so that the six samples can be compared under the condition of equal electric field and thickness (Tsuji and Hess 1986). At 555 nm (Fig. 3 a) the sign of ΔA changes twice with increasing frequency; at ≈ 0.1 Hz and at ≈ 100 kHz except for the sample B-4. This suggests that, except for the purely blue bacteriorhodopsin, there are at least three processes involved in the field-induced conformational change. At 646 nm (Fig. 3 b) a minimum in the amplitude is observed at ≈ 100 Hz for the samples A-1, B-1, and B-2, supporting the existence of three processes.

Figure 5 shows the wavelength dependence of the steady state of the absorbance change due to the alternating fields with frequencies 10 Hz and 100 kHz for the six samples. The amplitude is also normalized by the inverse of the film thickness. At 10 Hz a positive peak at 550 nm and a negative peak at 640 nm are observed for all six samples. However, if one compares the results of samples A-1 and B-1 or those of samples A-2 and B-3, the amplitude is found to depend on the orientation of the fragments; the amplitude for the oriented samples is smaller than that for the unoriented samples. At 100 kHz signals are very small

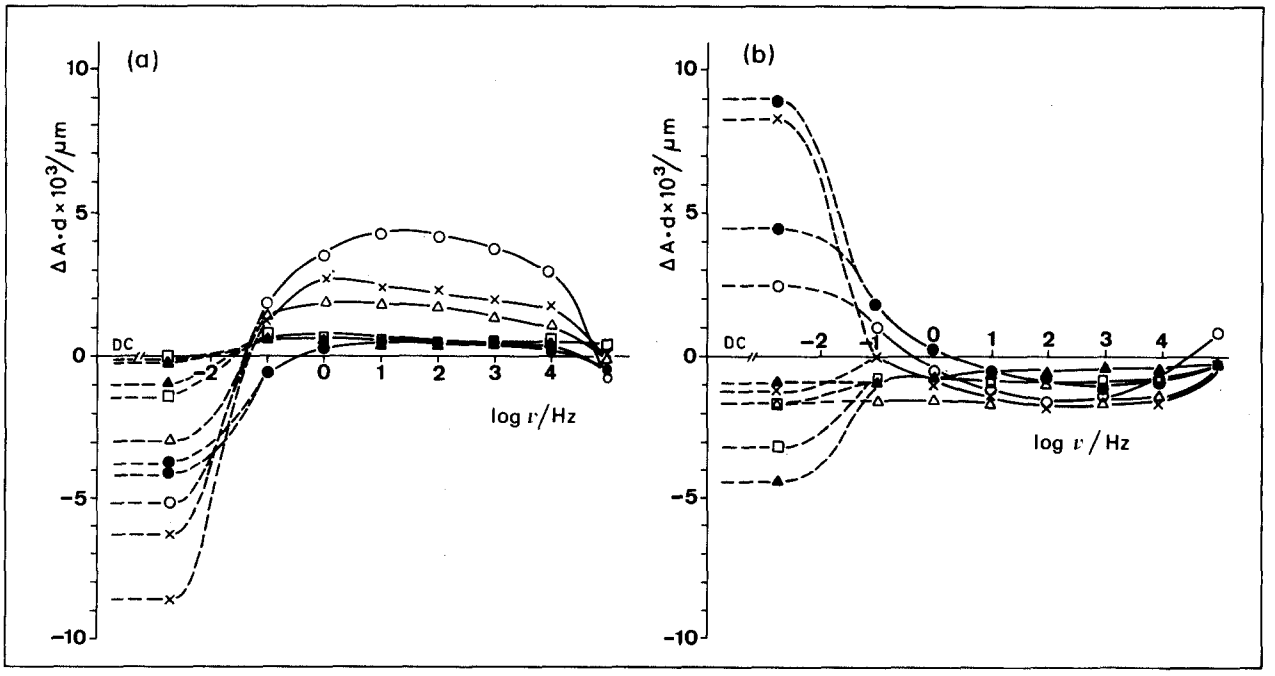

Fig. 3 a and b. Frequency dependence of the steady state of the electric field induced absorbance change for the six types of samples at $\lambda = 555$ nm (**a**) and $\lambda = 646$ nm (**b**). Amplitudes are normalized by the inverse of the thickness of film. $E \approx \pm 300$ kV/cm. (○), A-1; (△), A-2; (●), B-1; (×), B-2, (▲), B-3; (□), B-4

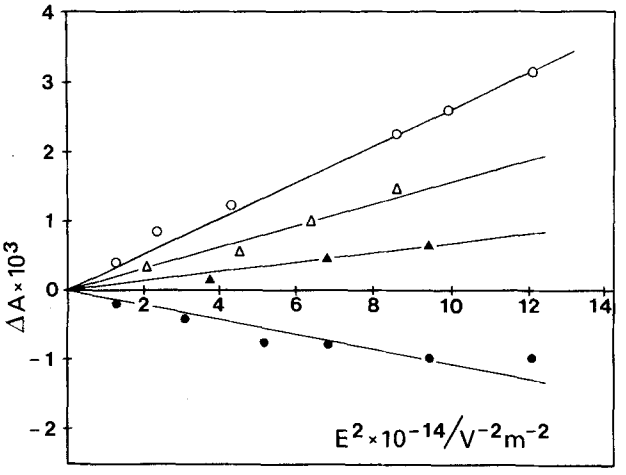

Fig. 4. Field strength dependence of the absorbance change of the purple membrane dried films at 10 Hz. ○, A-1, $\lambda = 555$ nm; △, B-2, $\lambda = 555$ nm; ▲, B-4, $\lambda = 555$ nm; ●, A-1, $\lambda = 646$ nm

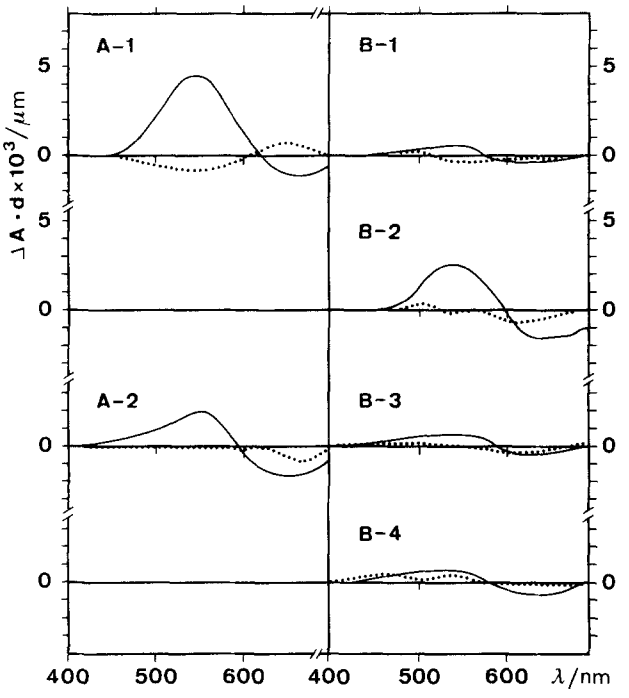

Fig. 5. Wavelength dependence of the steady state of the electric field induced absorbance change for the six types of samples due to the alternating fields with frequencies 10 Hz (*full line*) and 100 kHz (*dotted line*). Amplitudes are normalized by the inverse of the thickness of films. $E \approx \pm 300$ kV/cm

except for sample A-1 which shows a difference spectrum qualitatively similar to that due to DC fields (see Fig. 5 in Part I). In contrast to the DC field effects no dichroism is detected in the frequency range from 0.1 Hz to 100 kHz.

B. Dielectric measurements

In the majority of cases the frequency dependence of the complex permittivity shows two small and broad dispersion/absorption regions, one below 1 kHz and the other around 10–100 kHz. Examples of this behaviour are shown in Figs. 6a and b in terms of the measured sample capacitance and conductance. In the first case (Fig. 6a) the purple membrane film was placed on a Mylar sheet of 12 μm thickness. While the capacitance of a Mylar layer without membrane film does not show any appreciable frequency dependence (upper curve in Fig. 6a), there are two pronounced dielectric dispersion/absorption processes in the combined system. In the second example with much smaller surface area (Fig. 6b), prepared without Mylar sheet, a process around 1 kHz is clearly distinguishable. At higher frequencies there is only a slight frequency dependence which indicates a small dielectric contribution with a broad distribution of relaxation times. The steep slope at frequencies below 100 Hz is probably due to electrode polarization effects. The existence of two relaxation processes in the investigated frequency range supports the results shown in Fig. 3 – the sign of ΔA changes at $\approx$0.1 Hz and at $\approx$100 kHz.

No evaluation of absolute values of the permittivity is undertaken for the following reasons: Other than in the optical measurements, where only a small area of the sample is investigated (the area where the monitoring light passes is about 1 mm^2 of the total area of 280 mm^2), the dielectric measurements reflect the properties of the whole samples. It turns out that the sample capacitance and conductance are highly dependent on the effective surface area of the spattered electrodes. Since the formation of small cracks in the film is unavoidable during the drying procedure, small parts of the sample may not give any contribution. This results in a reduction of the sample capacitance that cannot be quantified at this stage of our investigations. In a few cases the suspension was dried on a thin Mylar film, which shows qualitatively the same frequency dependence of the permittivity, however, with larger values. This indicates that the presence of a Mylar sheet increases the effective area. We do not think that there is a remarkable contribution by polarization phenomena at the interface between the purple membrane film and Mylar layer, because in that case we would expect an additional pronounced frequency dependence at very low frequencies. The permittivity of spattered Mylar films of similar geometry without dried membrane films was determined with good precision (see Fig. 6a), which excludes other systematic errors in the measuring method.

Note that both the electric field strength ($\pm$6 kV/cm) and the field shape (sinus) in the dielectric measurements are different from those in the optical measurements ($\pm$300 kV/cm, rectangular). Nevertheless the results agree qualitatively with the results in the optical measurements and support the idea that

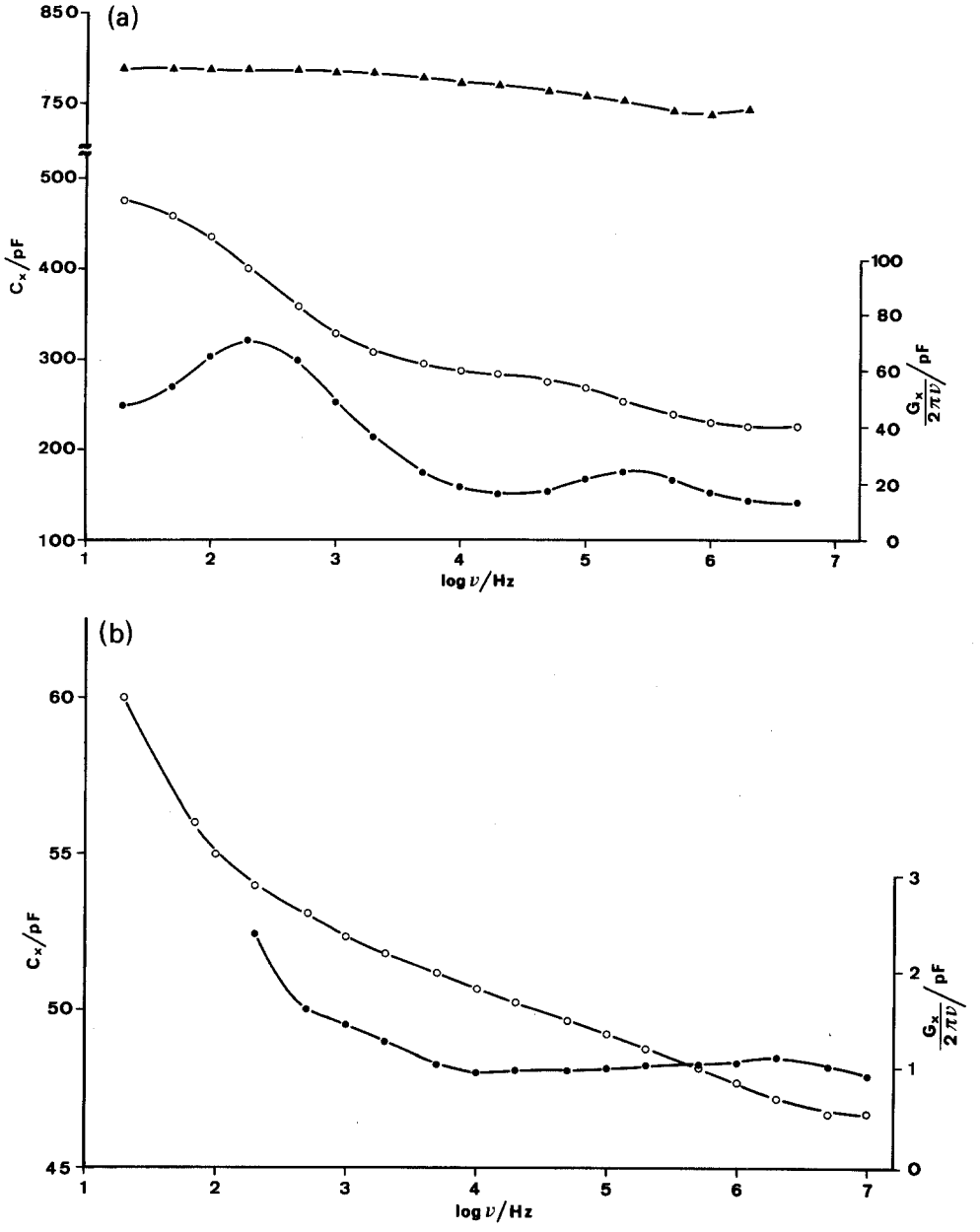

Fig. 6 a and b. Frequency dependence of the dispersive (o) and absorptive (•) parts of the admittance for: **a** the purple membrane film of the type A-1 ($d = 2$ μm, $S = 280$ mm^2) on a Mylar sheet ($d = 12$ μm), **b** the purple membrane film of the type A-1 ($d = 6$ μm, $S = 4$ mm^2). The frequency dependence of the dispersive part for Mylar sheet (▲, $d = 12$ μm, $S = 280$ mm^2) is also shown in **a**

essentially the same kind of conformational change is already induced at 6 kV/cm, at least to some extent. This is consistent with the finding that ΔA at 10 Hz is proportional to E^2.

Discussion

The results mentioned above combined with the results in Part I allow us to propose a cyclic reaction model for the conformational change of bacteriorhodopsin induced by the externally applied electric field. The reaction schemes for purple and blue bacteriorhodopsin are given in Figs. 7 a and b, respectively. (Note that the intermediary samples in the blue purple transition, B-2 and B-3, also show their own cyclic reactions).

According to the three processes, purple bacteriorhodopsin changes its conformation through intermediates X_1 and X_2, reaching a steady state X_3. After removing the field, it returns to the original purple bacteriorhodopsin through at least one intermediate, X_4, which is suggested by the biphasic decay process found in DC measurements.

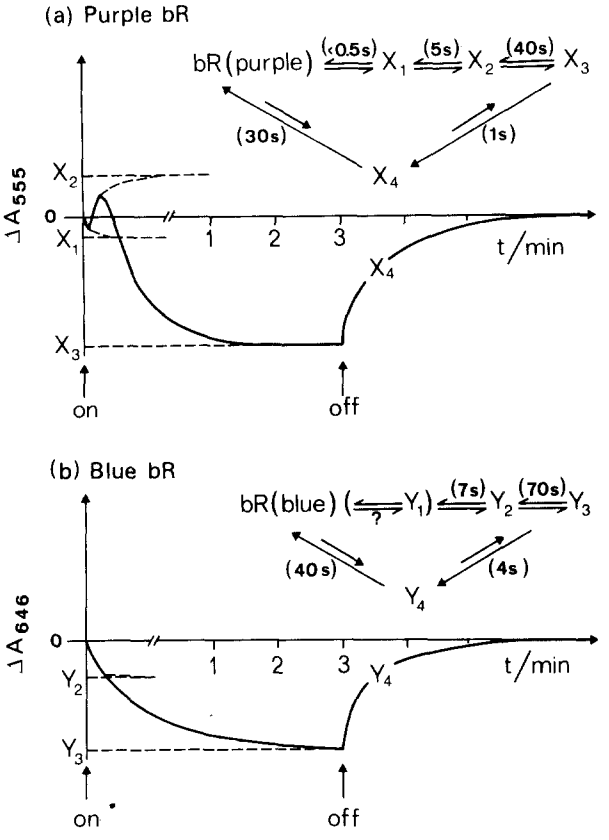

Fig. 7a and b. Reaction cycles of purple (**a**) and blue bacteriorhodopsin (**b**) due to the externally applied electric field

The time constant for the formation of the first intermediate X_1 is <5 s. This intermediate may be related to the dielectric relaxation process in the frequency range higher than 10–100 kHz, that is, an induced dipole system which can follow the field alternation of every 5 μs is involved. The difference spectrum of X_1 (spectrum for the sample A-1 at 100 kHz in Fig. 5) is qualitatively similar to that of the steady state X_3, indicating that bacteriorhodopsin changes its colour slightly in the direction from purple to blue at the beginning of the conformational change. This is probably due to a fast charge separation (within 5 μs) of ion pairs in the vicinity of retinal.

The time constant for the formation of the second intermediate X_2 is about 5 s. From the frequency which gives the maximum absorbance change we can estimate that the induced dipole system for this change follows the field alternation of every 50 ms (maximum at 10 Hz in Fig. 3). It may correspond to the relaxation process in the lower frequency range. The difference spectrum of X_2 (spectrum for the sample A-1 or B-1 at 10 Hz in Fig. 5) recalls the transition from the dark-adapted to the light-adapted bacteriorhodopsin – the 13-*cis* to all-*trans* isomerization of retinal (Oesterhelt et al. 1973). However, the wavelength of the maximum in the difference spectrum for X_2 is shorter than that for the dark-light transition in the dried film equilibrated with 43% relative humidity (Korenstein and Hess 1977a). Therefore, if isomerization took place during formation of X_2, it should be different from the 13-*cis*–all-*trans* isomerization.

The steady state X_3 depends on the field direction, as described in Part I. The electric field directed from the intracellular to the extracellular side of the membrane causes a spectrum change, similar to that in purple-blue transition by cation depletion (Kimura et al. 1984; Kohl et al. 1984). The time constant is ≈ 30 s.

When the electric field is applied to blue bacteriorhodopsin, the conformation is changed through, at least, one intermediate, Y_2 – neither the optical measurements nor the dielectric measurements clearly show the existence of the "first" intermediate Y_1 which may correspond to X_1 in the case of purple bacteriorhodopsin. The time constant for the formation of Y_2 is about 7 s. This intermediate is caused by an induced dipole system the relaxation time of which is similar to that of X_2 for purple bacteriorhodopsin. The difference spectrum for Y_2 (spectrum at 10 Hz for the sample B-4 in Fig. 5) is qualitatively similar to that of the blue-purple transition induced by adding cations; the colour of blue bacteriorhodopsin slightly shifts to purple before the DC steady state, Y_3, is established. It suggests that some cations which are depleted from the colour-sensitive site and which still remain in the blue dried film are re-injected by the electric field. As we mentioned in Part I, in the steady state the retinal may be removed from the protein. When the electric field is turned off, the retinal is rebound to protein and the conformation of the bacteriorhodopsin is changed back to that of the original blue state through one intermediate, Y_4.

As an additional result, we found a quantitative difference in the difference spectra between the oriented and unoriented samples. The conformational change caused by a DC field is dependent on its direction with respect to the orientation of the membrane (Part I). Since the intracellular side of the purple membrane is electrically and structurally different from the extracellular side, this is to be expected. On the other hand, if the duration of the alternating field is long enough compared to its period, the total time when the electric field is applied from the intracellular side to the extracellular side should be the same as in the case of the opposite direction, whatever the orientation of the membrane is. Consequently, for each membrane fragment the accumulated signal should be independent of the membrane orientation, as shown schematically in Fig. 8. In Fig. 8(A) the fragments *a* and *b* orient in opposite directions, and in Fig. 8(B) they orient in the same direction. For both cases the accumulated signal for a single fragment is the same. Furthermore, if there

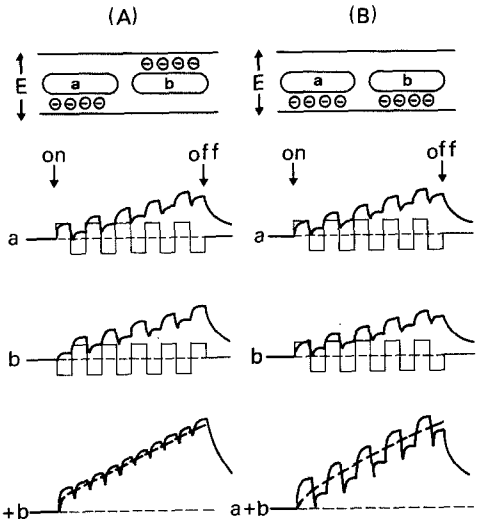

Fig. 8A and B. Scheme for accumulation of the optical signal due to the alternating field for the unoriented sample (**A**) and the oriented sample (**B**). See text

is no interaction between the fragments *a* and *b*, the total signal which we observe should be simply a sum of the signal from each single fragment, resulting in identical total signals for the unoriented and the oriented sample. However, if we compare the optical signal for the unoriented sample A-1 at 10 Hz with that for the oriented sample B-1, or that for the sample A-2 with B-3 in Fig. 5, it turns out that the amplitudes for the unoriented samples are larger than for the oriented samples. This suggests that electrical interaction between the surfaces of the fragments is important.

As a concluding remark, we compare the field-induced cyclic reaction of bacteriorhodopsin with the photocycle. First of all, since no maximum around $\lambda = 410$ nm is observed in the difference spectra in the frequency range from 0.003 Hz to 100 kHz (see Fig. 5), no deprotonation of the Schiff's base is indicated, that is, the *M* intermediate of the photocycle is not formed. Isomerization of the retinal may take place, which should be, however, different from the 13-*cis*–all-*trans* isomerization. Difference spectra similar to that of the purple-blue or the blue-purple transition suggest that cations are reversibly removed from the colour sensitive site. The time scale of the field-induced reaction cycle (in $\approx 50\%$ room humidity) is comparable to that of the photocycle of purple membrane films in 43% room humidity (Kornstein and Hess 1977 b).

Thus, the field-induced reaction and the photocycle are to some extent related to each other. This is further supported by the observation of the field-induced pH change in the purple membrane suspension (Tsuji and Neumann 1981 b), which shows the opposite sequence of pH-increase and -decrease to the light-induced pH change (Govindjee et al. 1980). Since the photoreaction of bacteriorhodopsin concomitant with proton pumping (Lozier et al. 1975) leads to an increase of the membrane potential (Michel and Oesterhelt 1976) it can be concluded that the electric potential across the membrane is essential for the conformation of bacteriorhodopsin, whether it is an intrinsic membrane potential or an externally applied one.

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