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CONFORMATIONAL FLEXIBILITY OF MEMBRANE PROTEINS IN ELECTRIC FIELDS

I. ULTRAVIOLET ABSORBANCE AND LIGHT SCATTERING OF BACTERIORHODOPSIN IN PURPLE MEMBRANES

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Bacteriorhodopsin of halobacterial purple membranes exhibits conformational flexibility in high electric field pulses ($1-30\times10^5$ V m^{-1}, 1–100 μs). High-field electric dichroism data of purple membrane suspensions indicate two kinetically different structural transitions within the protein; involving a rapid (≈ 1 μs) concerted change in the orientation of both retinal and tyrosine and/or tryptophan side chains concomitant with alterations in the local protein environment of these chromophores, as well as slower changes (≈ 100 μs) of the microenvironment of aromatic amino acid residues concomitant with pK changes in at least two types of proton-binding sites. Light scattering data are consistent with the maintenance of the random distribution of the membrane discs within the short duration of the applied electric fields. The kinetics of the electro-optic signals and the steep dependence of the relaxation amplitudes on the electric field strength suggest a saturable induced-dipole mechanism and a rather large reaction dipole moment of 1.1×10^{-25} C m ($=3.3\times10^4$ debye) per cooperative unit at $E=1.3\times10^5$ V m^{-1}, which is indicative of appreciable cooperativity in the probably unidirectional transversal displacement of ionic groups on the surfaces of and within the bacteriorhodopsin proteins of the membrane lattice. The electro-optic data of bacteriorhodopsin are suggestive of a possibly general, induced-dipole mechanism for electric field-dependent structural changes in membrane transport proteins such as the gating proteins in excitable membranes or the ATP synthetases.

1. Introduction

Evidence is accumulating that proteins are dynamic structures even in the solid form of a protein crystal [1–3]; also, membrane proteins embedded in the lipid matrix of biomembranes may exhibit not only lateral motion but also intrinsic conformational flexibility. Despite the lattice-like pattern observed in the purple membranes of halobacteria [4] there is conformational mobility within bacteriorhodopsin, permitting, for instance, *trans-cis* isomerization of the retinal part [5]. The close contacts between the bacteriorhodopsins in the lattice prevent major movements of the bacteriorhodopsins within the membrane; but transversal mobility along the membrane normal is apparent from enzymatic digestion studies [6].

As to membrane proteins in general, it is important to realize that all biological membranes appear to have a membrane electric potential (difference) under in vivo conditions. In halobacteria, the 'resting' membrane potential, $\Delta\psi$, is about -60 mV [7], taking the cell outside as a reference of zero electric potential. Assuming a thickness, d, of about 5×10^{-7} cm (50 Å) for the membranes, this potential difference formally corresponds to an average electric field strength, $\bar{E}$, across the membrane of $\bar{E}=\Delta\psi/d=1.2\times10^7$ V m^{-1} (120 kV cm^{-1}); the field is (for positive charges) directed from the outside to the cell interior. Besides the stationary field, the light-induced proton pumping leads to a hyperpolarizing increase in the

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absolute value of the membrane potential by up to 10–40 mV [7], or to an increase in the average value of the membrane's electric field by $\Delta\bar{E} \approx 2$–7×10^6 V m^{-1} (20–70 kV cm^{-1}).

Since bacteriorhodopsin is polyionic it is likely that the protein structure (and thus photocycle and proton transport) is directly affected by the membrane electric field. When purple membranes are isolated and studied in aqueous suspension the intrinsic natural electric field is largely reduced; only the field contributions from the asymmetrically distributed surface charges [8] and dipoles remain. If the decoupling of the purple membrane patches from the bacterial cell membrane has caused structural changes in bacteriorhodopsin, the application of external electric fields may partially reverse these conformational relaxations.

Indeed, it has recently been found that short electric field pulses of relatively high electric field strength (1–30×10^5 V m^{-1} and 1–100 μs duration) cause structural changes within the bacteriorhodopsin of purple membrane suspensions [9]; the field-induced transitions comprise two types of changes: (1) restricted rotational displacement of the retinal chromophore by an angle of 0.35 rad or greater ($\geq 20°$), unidirectionally toward the membrane normal and (2) alterations of the pK values of at least two types of proton-binding sites [10]. Quantitative analysis of electro-optic data had established that an induced-dipole mechanism is operative. The numerical values refer, however, only to models which assume that the electric dipole axis is fixed relative to the optical transition moment of the retinal chromophore [9]. It was also found that the electric dichroism in the visible absorbance range and the pH changes are not equivalent as an indicator of the field-induced conformational changes in bacteriorhodopsin [10].

In the present study, it is demonstrated that field-induced absorbance changes at 280 nm and light scattering changes at 700 nm indicate structural changes in the protein moiety. These conformational transitions occur in at least two kinetically different phases. The analysis of the optical transition curves yields estimates of the electric reaction dipole moments. The large numerical values of the dipole moment differences of the two phases and the sigmoidal on-set kinetics suggest cooperative interactions. In part II of this series [11], the electro-optic results are analyzed in terms of a multi-step reaction model involving two-dimensional cooperativity.

2. Experimental

2.1. Materials

Purple membranes isolated from the S9 strain of *Halobacterium halobium* were a gift from Professor D. Oesterhelt and Dr. Michel. The concentration of bacteriorhodopsin in purple membrane suspensions was determined on the basis of an extinction coefficient of $\epsilon_{570} = 63000$ M^{-1} cm^{-1} at 570 nm [12]. For the electro-optic measurements, the purple membranes are suspended in (reflux-multiple) distilled water of conductivity 0.9 μS cm^{-1}. The bacteriorhodopsin concentration of the suspension is 5.1×10^{-6} M; the optical density, OD, at 570 and 280 nm is $OD_{570} = 0.31$ and $OD_{280} = 0.59$ per cm, respectively. As is well known, the optical density comprises both absorbance, A, and scattering effect, S, according to

$$OD = A + S \quad (1)$$

While the light scattering at 570 nm is negligible for this concentration, S at 280 nm constitutes a major portion to OD. Under the present experimental conditions, we have $OD_{280}/OD_{570} = 0.59/0.31 = 1.9$, while the ratio of the extinction coefficients was reported to be $\epsilon_{280}/\epsilon_{570} = 1.2$ [13]. Throughout the experiments bacteriorhodopsin is in the light-adapted form.

2.2. Electric field-induced optical changes

The electro-optic measurements were carried out with an electric relaxation spectrometer [14]. Collimated light from a 200 W mercury lamp passed through a Schoeffel monochromator and a Nicoll polarizer which can be rotated from $\sigma = 0$ to $\sigma = 2\pi$ with respect to the direction of the external electric field, thus providing polarized light for the indication of rotational and chemical changes in the sample cell.

Rectangular electric pulses up to 25×10^5 V m^{-1} with pulse duration of 1–100 μs were applied to the sample solution. The light transmitted through the cell was detected by a photomultiplier head (1P28A; 10–90% rise time of 8 ns), amplified and displayed on an oscilloscope. The optical detection device is a development by C.R. Rabl.

The optical density change per cm, ΔOD_σ, at the light polarization mode, σ, with respect to the electric field direction is calculated from the light transmittance change, ΔI, according to

$$\Delta OD_\sigma = -\frac{1}{l}\log(1 + \Delta I_\sigma / I_\sigma) \tag{2}$$

where I_σ is the transmitted light in the absence of the electric field and l (in cm) the light path length of the sample cell.

For measurements of the electric field-induced light scattering change, ΔI_σ, at 700 nm, the photomultiplier (EMI 9558 QA; 10–90% rise time of 500 ns [15]) was set at $\pi/2$ with respect to the light source. Polarizers were inserted both between the monochromator and the sample cell and between the sample cell and the photomultiplier. The field-induced light scattering change is defined as

$$\Delta I_s = I_s^E - I_s^0 \tag{3}$$

where I_s^E and I_s^0 are the intensities of the scattered light in the presence and absence of the electric field, respectively.

All measurements were carried out at 293 K. The temperature increase due to the Joule heating causes only negligible small contributions to ΔOD [9].

3. Theoretical background

As outlined previously [9], the electric field-induced absorbance changes ΔA_σ may be viewed in terms of two types of contributions, a linear dichroism part, $\Delta A_\sigma^{(rot)}$, depending on σ and a chemical part, $\Delta A^{(ch)}$ which is independent of the polarization angle:

$$\Delta A_\sigma = \Delta A_\sigma^{(rot)} + \Delta A^{(ch)} \tag{4}$$

where, analogous to eq. 3, the definition

$$\Delta A_\sigma = A_\sigma^E - A_\sigma^0 \tag{5}$$

holds [16,17].

3.1. Chemical transformations in an electric field

It is well known that structural transitions of the type

$$B_0 \rightleftharpoons B_1 \tag{6}$$

induced by an electric field E, at constant pressure P and constant (Kelvin) temperature T, may be described by [18–20]

$$\left(\frac{\partial \ln K}{\partial E}\right)_{P,T} = \frac{\Delta M}{RT}. \tag{7}$$

where $K = [B_1]/[B_0]$ is the (apparent) equilibrium constant (concentration ratio) and R the gas constant. The quantity ΔM is the (apparent) molar reaction dipole moment; it is usually the difference between the 'field-parallel' components of the macroscopic molar dipole moments of the two conformations considered: $\Delta M = M_1 - M_0$ (see part II of this series [11]). Since K is related to the mole fraction $\theta = [B_1]/([B_1] + [B_0])$ of B by

$$K = \theta/(1 - \theta) \tag{8}$$

eq. 7 can be rewritten in terms of θ. At a given field strength the 'slope' of the transition curve, $\theta(E)$, is given by:

$$\left(\frac{\partial \theta}{\partial E}\right)_{P,T} = \theta(1-\theta)\frac{\Delta M}{RT} \tag{9}$$

Note that $\partial\theta/\partial E = (\partial\theta/\partial \ln K)(\partial \ln K/\partial E)$ [17]. As seen in eq. 9 an electric field may change the degree of structural transition if $\Delta M \neq 0$ and $0 < \theta < 1$. Note that θ refers to an average over all randomly distributed membrane fragments [20].

When the conformations B_0 and B_1 have different (average) extinction coefficients $\bar{\epsilon}_0$ and $\bar{\epsilon}_1$, respectively, the electric field-induced chemical shift may be monitored as an absorbance change. Per cm of light path we obtain

$$\Delta A^{(ch)} = (\bar{\epsilon}_1 - \bar{\epsilon}_0) c \Delta\theta \tag{10}$$

where $c = [B_0] + [B_1]$ is the total concentration of B.

When θ already has a finite value θ^0 at $E = 0$, $\Delta\theta = \theta - \theta^0$. The field-induced absorbance change $\Delta A^{(ch)}$ may now be normalized by the saturation

value $\Delta A_{sat}^{(ch)}$ where $\theta = 1$. The chemical transition is then represented by the transition factor, defined as

$$\phi^{(ch)} = \frac{\Delta A^{(ch)}}{\Delta A_{sat}^{(ch)}} = \frac{\theta - \theta^0}{1 - \theta^0} \tag{11}$$

and the differentiation of eq. 11 leads to

$$\left(\frac{\partial \theta}{\partial E}\right)_{P,T} = (1 - \theta^0)\left(\frac{\partial \phi^{(ch)}}{\partial E}\right)_{P,T} \tag{12}$$

Now, the general expression for ΔM is derived from eqs. 9 and 12:

$$\Delta M = RT \frac{1 - \theta^0}{\theta(1 - \theta)}\left(\frac{\partial \phi^{(ch)}}{\partial E}\right)_{P,T} \tag{13}$$

Note that for the limiting case $\theta^0 \ll 1$, we have the approximation $\theta \cong \phi^{(ch)}$. For this condition eq. 13 permits an estimate of ΔM at the respective field strength from the slope of the transition curve: in particular, around the inflection point where a graphical slope determination is relatively reliable compared to the other parts of the transition curve.

3.2. Electric field-induced orientation

Dipolar species tend to orient in the electric field, resulting in (linear) electric dichroism. For a system which is axially symmetric and which has only a small optical anisotropy, the dependence of the absorbance change on the polarization mode σ, due to the orientation, is given by:

$$\Delta A_\sigma^{(rot)} = C(3\cos^2\sigma - 1). \tag{14}$$

where C is a constant [21].

The electric dichroism is generally defined as

$$\Delta A = A_\parallel - A_\perp = \Delta A_\parallel - \Delta A_\perp \tag{15}$$

where the ΔA_σ terms are measured optical signals according to eq. 2 when $|\Delta S_\sigma| \ll |\Delta A_\sigma|$. The parallel mode $\Delta A_\parallel$ refers to $\sigma = 0$ and the perpendicular mode $\Delta A_\perp$ refers to $\sigma = \pi/2$. Introducing eq. 4 into eq. 15, we readily see that the classical expression for the dichroism $\Delta A = \Delta A_\parallel^{(rot)} - \Delta A_\perp^{(rot)}$ is identical with the definition in eq. 15.

3.3. Light scattering anisotropy and depolarization

When macromolecules or particles which are smaller than the wavelength are spherically symmetric and optically homogeneous, the light scattered at $\pi/2$ with respect to the incident light is completely vertically polarized [22]. For ideally isotropic particles, the intensities of the scattered light are thus:

$$I_s(H_h) = I_s(H_v) = I_s(V_h) = 0,$$
$$I_s(V_v) \neq 0, \tag{16}$$

where H and V refer to the horizontal and vertical components of the scattered light (perpendicular and parallel to the electric field in the experimental set-up, see fig. 3), and h and v denote the horizontal and vertical components of the incident light. On the other hand, when the scattering particles are not spherically symmetric or are optically inhomogeneous, the light scattered at $\pi/2$ has an additional horizontal component, and we obtain:

$$I_s(V_v) \neq 0,$$
$$I_s(H_v) = I_s(V_h) = 0,$$
$$I_s(H_h) \neq 0. \tag{17}$$

Therefore, when depolarization effects are found which correspond to eq. 17, shape asymmetry of the particles or optical inhomogeneity is present.

4. Results

Fig. 1 shows typical optical signal changes at 280 nm induced by an electric impulse in a purple membrane suspension at the light polarization modes $\sigma = 0$, $\sigma = \pi/2$ and at the angle $\sigma = 0.73$ rad (41.5°). At any polarization direction except $\sigma = 0.73$, the optical density change consists of two clearly discernible components, a fast mode (phase I; the OD increases for $0 \leq \sigma \leq 0.73$ and decreases for $0.73 < \sigma \leq \pi/2$) and a slow relaxation mode (phase II) which does not reach a clear-cut steady state even when at $E = 20 \times 10^5$ V m^{-1} the field duration is as long as $\Delta t = 100$ μs. At $\sigma = 0.73$, only the slower component is observed. Both the amplitudes and the kinetics of the slower component are independent of the polarization angle. The fast relaxation mode (phase I) shows a sigmoidal onset (corresponding to a 'delay time' of about 200 ns) and saturates at about 6 μs at

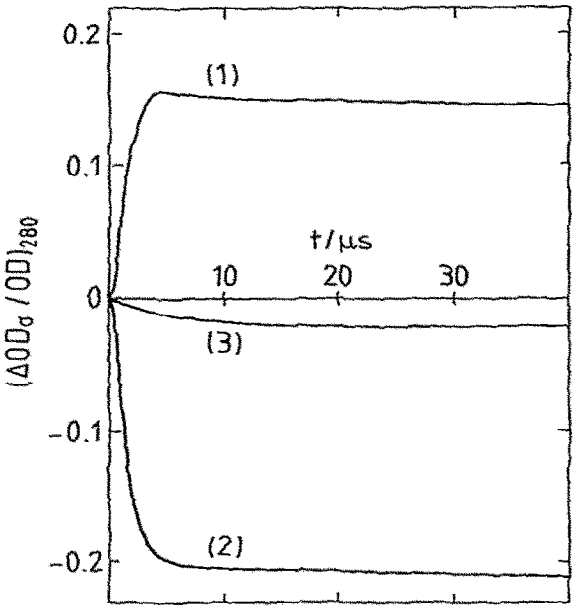


Fig. 1. Relative optical density changes $(\Delta OD_\sigma/OD)_{280}$ at $\lambda = 280$ nm induced by a 'rectangular' electric field, $E = 15.4 \times 10^5$ V m^{-1} ($= 15.4$ kV cm^{-1}), applied at time $t = 0$ for 40 μs, to a suspension of purple membrane fragments at 293 K; the bacteriorhodopsin concentration is $c = 5.1 \times 10^{-6}$ M in H_2O. $OD_{280} = 0.59$. The curves refer to three polarization modes of the polarized light relative to the E direction: (1) $\sigma = 0$, parallel mode; (2) $\sigma = \pi/2$, perpendicular mode; and (3) $\sigma = 0.733$ rad (where the rapid phase (I) disappears). Note on the sigmoidal on-set in 1 and 2.

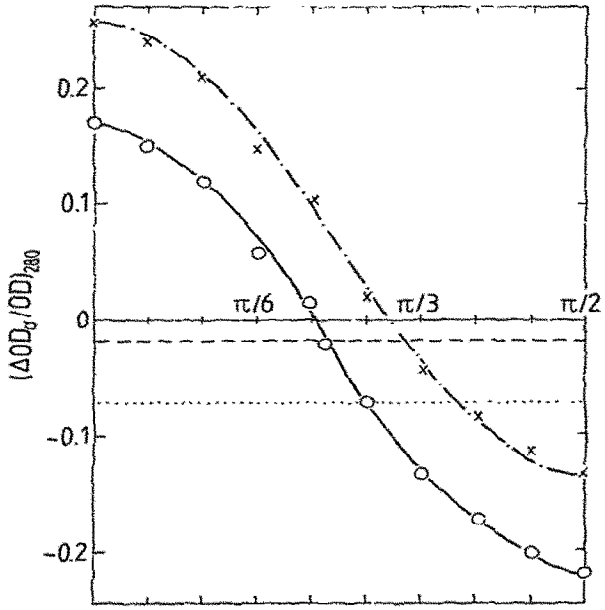


Fig. 2. Relative OD changes at 280 nm $(\Delta OD_\sigma/OD)_{280}$ at $t = 40$ μs, after applying (at $t = 0$) a rectangular electric field of $E = 15.4 \times 10^5$ V m^{-1} to a purple membrane suspension of $OD_{280} = 0.59$ at 293 K, as a function of the light polarization angle σ. (○) Measured OD change; (x) amplitude of the rapid phase $\Delta A_{280}^{(rot)}(I)$ (see fig. 1); (——— and –·–·–) calculated according to eq. 14; (·····) amplitude of the rapid phase $\Delta OD_{280}^{(ch)}(I)$; and (- - - - -) amplitude of the slow phase $\Delta OD_{280}^{(ch)}(II)$.

15×10^5 V m^{-1}. Whereas the amplitude of the faster component depends on the polarization direction, the time constants are independent of σ.

The total optical density change induced by the electric field is therefore expressed as

$$\Delta OD_\sigma = \Delta OD_\sigma(I) + \Delta OD(II) \quad (18)$$

According to eq. 4, $\Delta OD_\sigma(I)$ may have two parts, one depending on σ (the rotational contribution) and the other one independent of σ (the chemical contribution). Both parts may generally contain scattering contributions. Thus:

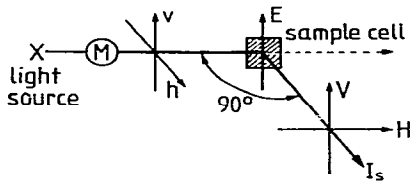
$$\Delta OD_\sigma(I) = \Delta OD_\sigma^{(rot)}(I) + \Delta OD^{(ch)}(I) \quad (19)$$

As shown in fig. 2, $\Delta OD_\sigma^{(rot)}(I)$ follows eq. 14, indicating that the system is axially symmetric and that the scattering contribution is independent of σ, or $\Delta S_\sigma^{(rot)} = 0$. Therefore, the rotational contribution of the OD change reflects solely that of the absorbance change. Hence,

$$\Delta OD_\sigma^{(rot)}(I) = \Delta A_\sigma^{(rot)}(I) \quad (20)$$

The time constants characterizing the time courses of $\Delta A_\sigma^{(rot)}(I)$ are independent of σ.

Fig. 3 summarizes the results of the light scattering experiments; compiled are the numerical values of I_s^0, the light scattered at $E = 0$ and those of the field-induced changes ΔI_s at $E = 15.4 \times 10^5$ V m^{-1} and at a field duration of $\Delta t = 40$ μs. The numerical values are normalized to $I_s^0(V_v)$. The finite values of I_s^0 for the polarization modes



	V_v	H_v	V_h	H_h
(a) $I_s / I_s(V_v)$	1.0	0	0	0
(b)	1.0	≠0	≠0	≠0
(c) $I_s^0 / I_s^0(V_v)$	1.0	≠0	≠0	0.38
(d) $\Delta I_s / I_s^0(V_v)$	1.3	0	0	0.06

Fig. 3. Intensities (I_s^0) of the scattered light ($\pi/2$) at $E = 0$ and the electric field-induced changes (ΔI_s) at the polarization modes v and h of the incident light and V and H of the scattered light, relative to the E direction and normalized with respect to $I_s^0(V_v)$. Case a refers to ideally isotropic, optically homogeneous spheres, b refers to anisotropic, asymmetric and/or optically inhomogeneous particles (see eqs. 16 and 17 of the text). Cases c and d refer to a suspension of purple membrane discs at 293 K (c) at $E = 0$ and (d) at $E = 15.4 \times 10^5$ V m^{-1} ($\Delta t = 40$ μs).

H_v, V_h and H_h are consistent with the anisotropic shape of the purple membrane fragments. The field-induced scattering changes $\Delta I_s(H_v)$ and $\Delta I_s(V_h)$ are zero and $\Delta I_s(H_h)$ is very small compared to $\Delta I_s(V_v)$. These facts suggest that the electric fields of the short durations applied here do not cause any additional anisotropy: either any change in the random orientation of the scattering particles, or any optical inhomogeneity (see eqs. 16 and 17). Therefore, optical contributions from fragment orientations are negligibly small because the statistical distribution of the purple membrane discs does not change within the small pulse durations (see also refs. 9 and 10).

Fig. 4 shows the normalized time courses of three optical signals: (1) $\Delta A_{280}^{(rot)}$(I), (2) ΔOD_{280}(II) and (3) $\Delta I_s(V_v)$, for both the 'field-on' and the 'field-off' responses. It is seen that $\Delta A_{280}^{(rot)}$(I) reaches a steady state already within 6 μs whereas ΔOD_{280}(II) still continues to change. The field-off relaxation of ΔOD_{280}(II) can be analyzed as a single-exponential function with a relaxation time

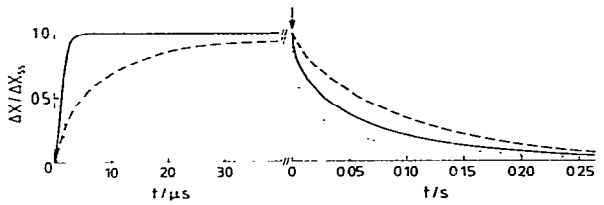


Fig. 4. Time courses of the electric dichroism (eq. 15 of the text) and of the chemical changes, ΔX_λ, induced by an electric field of $E = 15.4 \times 10^5$ V m^{-1} in a purple membrane suspension at 293 K, normalized to the respective steady-state values, ΔX_{ss}, in the field ($0 \leq t \leq 40$ μs) and after switching off the field (arrow). $\Delta X/\Delta X_{ss}$ refers to: (a) (———) $\Delta A_{280}(\frac{1}{2})/(\Delta A_{280}(I))_{ss} = \Delta A_{280}^{(ch)}(I)/(\Delta A_{280}^{(ch)}(I))_{ss}$ which is about the same as the corresponding terms for ΔA_{565} and $\Delta A_{565}^{(ch)}$ in ref. 9; (b) (- - - - - -) $\Delta OD_{280}^{(ch)}(II)/(\Delta OD_{280}^{(ch)}(II))_{ss}$; (c) (· · · · · ·) $\Delta I_s(V_v)/(\Delta I_s(V_v))_{ss}$ at 700 nm.

of 100 ms under the given conditions. On the other hand, the rapid mode $\Delta A_{280}^{(rot)}$(I) reflects a continuous spectrum of processes with relaxation times between 100 μs and 100 ms; the average relaxation time is about 60 ms.

It is important to mention that the time course of the retinal displacement indicated by $\Delta A_{565}^{(rot)}$ is the same as that of $\Delta A_{280}^{(rot)}$(I).

The field-on relaxation of $\Delta I_s(V_v)$ may be approximated as a combination of $\Delta A_{280}^{(rot)}$(I) and ΔOD_{280}(II). After switching off the field, $\Delta I_s(V_v)$

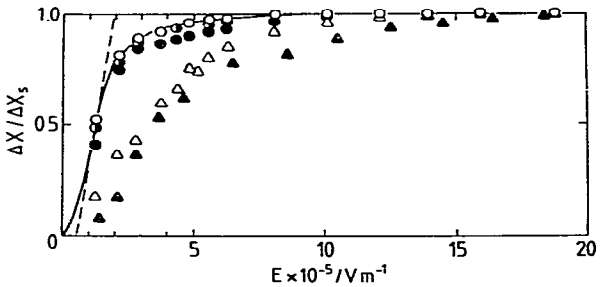


Fig. 5. Amplitudes $\Delta X(E)$ of the electric dichroism, ΔA (eq. 15 of the text), and of the chemical changes induced by E in a purple membrane suspension at 293 K, as a function of the external electric field, normalized to the respective apparent saturation values ΔX_s (at $E = 20 \times 10^5$ V m^{-1}). (○) ΔA_{565}, (●) ΔA_{280}(I), (◑) ΔA_{220}(I), (△) $\Delta OD_{280}^{(ch)}$(II), (▲) Δ pH$_{max}$ in ref. 10. The lines refer to the data evaluation according to eq. 13 of the text.

decays faster than $\Delta A_{280}^{(rot)}(I)$; the average relaxation time is about 40 ms under the given conditions.

As shown in fig. 5, the normalized field strength dependences of $\Delta A_{\lambda}^{(rot)}(I)$ at the wavelengths $\lambda =$ 565, 280 and 220 nm are practically coincident. The field strength dependence of $\Delta OD_{280}(II)$ and that of the maximum pH change, ΔpH_{max}, caused by the electric pulse [10], both reach their saturation values at a higher electric field than $\Delta A_{280}^{(rot)}(I)$ and $\Delta A_{565}^{(rot)}$.

5. Discussion

The absorbance of the purple membranes at 565 nm is clearly associated with the retinal chromophore. According to Becher et al. [13], the absorbance at 280 nm is attributed to tyrosine and tryptophan residues ($\approx 90\%$) and to the retinal ($\approx 10\%$). Most of the tyrosine and tryptophan residues appear to be in the hydrophobic environment. If all tyrosine and tryptophan residues were in a hydrophilic environment, the observed absorbance would be smaller by approx. 20%.

5.1. Rapid mode (I)

In figs. 4 and 5 it is shown that both the kinetics and the field strength dependence of $\Delta A_{280}(I)$ and ΔA_{565} are the same, with respect to both the chemical and the rotational contributions. Therefore, the electric dichroism at both wavelengths appears to arise from one and the same mode of an overall molecular process. It was previously shown that this process involves the (restricted and asymmetric) rotation of the retinal chromophore by an angle of 0.35 rad or greater towards the membrane normal [9]. In order to specify further the optical changes it is remarked that at 280 nm, the ratio $\Delta OD_{280}^{(ch)}(I)/\Delta A_{280}^{(rot)}(I) = -0.18$, and at 565 nm the ratio $\Delta A_{565}^{(ch)}/\Delta A_{565}^{(rot)} = -0.04$. The larger absolute value of the ratio at 280 nm, therefore, indicates that there are either scattering contributions to $\Delta OD_{280}^{(ch)}(I)$ or/and changes in the environment of a tyrosine or a tryptophan residue (most probably Tyr-26 [23]), due to field-induced changes in the interaction with retinal.

As possible origin of the field-induced increases in the intensity of the scattered light at 700 nm, we may consider (1) a volume change of the membrane or a change in the refractive index of the purple membranes due to structural changes of bacteriorhodopsin, (2) an increase in the attractive force between particles (and subsequent pearl-chain-like aggregation) due to the increase in the electric dipole moments, (3) fragment orientation (the membrane normal oriented perpendicular to the field) [23]. As already mentioned in section 4, the field-induced light scattering changes ΔI_s are 'isotropic' in spite of the disc form of the particles. Alternatives 2 and 3 can therefore be excluded for the present experimental condition of high electric field strength and relatively short pulse durations. However, for longer pulse durations, changes in the light scattering [24], electric dichroism [25,26] and dielectric dispersion [27] have been interpreted in terms of fragment orientation.

5.2. Slow mode (II)

The slow optical density change $\Delta OD_{280}^{(ch)}(II)$ also reflects structural changes in the protein part of bacteriorhodopsin. However, at present, we cannot separate the contributions of the pure absorbance change and the scattering change. Therefore, it is presently not possible to estimate exactly the net number of tyrosine and tryptophan residues, which change from a hydrophobic to a hydrophilic environment under the influence of the electric field.

The displacement of the chromophores is most likely asymmetric. When the rotation towards the membrane normal is associated with the extinction coefficient difference $\bar{\epsilon}_1 - \bar{\epsilon}_0$ and that away from the normal with $\bar{\epsilon}_1' - \bar{\epsilon}_0$ the absorbance change is given by

$$\begin{aligned}\Delta A^{(ch)} &= [0.5(\bar{\epsilon}_1 - \bar{\epsilon}_0) + 0.5(\bar{\epsilon}_1' - \epsilon_0)]\, c\Delta\theta \\ &= [0.5(\bar{\epsilon}_1 - \bar{\epsilon}_1') - \bar{\epsilon}_0]\, c\Delta\theta \end{aligned} \quad (21)$$

If $\bar{\epsilon}_1' = \bar{\epsilon}_0$, as suggested by the linear dichroism of the retinal chromophore [9], then

$$\Delta A^{(ch)} = 0.5(\bar{\epsilon}_1 - \bar{\epsilon}_0)\, c\Delta\theta; \quad (22)$$

this means that in a random distribution of the chromophores only half of the total substance contributes to $\Delta A^{(ch)}$.

From fig. 2 and eq. 22, the maximum difference in the molar extinction coefficient at 280 nm is estimated to be

$$\Delta\epsilon_{280} = \frac{2}{c}\Delta A_{280}^{(ch)}(II) \geq \frac{2}{c}\Delta OD_{280}^{(ch)}(II)$$
$$\approx -4 \times 10^3\ M^{-1}\,cm^{-1};$$

i.e., $\bar{\epsilon}_0 > \bar{\epsilon}_1$ at 280 nm.

According to the values given by Donovan [27], this change in the extinction coefficient could account for the transition of 2–3 tryptophan ($2–3 \times 1600\ M^{-1}\,cm^{-1}$) or 5–6 tyrosine ($5–6 \times 700\ M^{-1}\,cm^{-1}$) residues from the membrane interior to the aqueous environment. The numerical values are upper limits for negligibly small scattering contributions to ΔOD_{280}.

The secondary structure (helical part) of bacteriorhodopsin seems to be hardly affected by the electric field, because $\Delta OD(II)/OD$ at 220–240 nm is much smaller than the OD changes in the near ultraviolet region (data not shown).

5.3. Reaction model

As discussed previously [10], intramolecular structural changes may involve small transverse displacements of some groups of bacteriorhodopsin, preferably along the direction of the membrane normal. Such transverse displacements are evident from enzymatic digestion studies; it was found that some parts of the protein are more exposed to the outside when the membrane is in the proton-pumping state [6].

The appearance of chemical contributions $\Delta A_{565}^{(ch)}$, $\Delta OD_{280}^{(ch)}(I)$ and $\Delta OD_{280}^{(ch)}(II)$ to the field-induced optical density changes clearly demonstrates that high electric pulses indeed cause structural changes within the protein. The similarity of the electric field strength dependence of $\Delta OD_{280}(II)$ and of the pH changes, ΔpH_{max}, indicates that the structural changes observed at 280 nm are also those which lead to changes in the pK values of at least two types of proton-binding sites [10].

Recalling fig. 4, it is stressed that the retinal chromophore orientation reflected in $\Delta A_{565}^{(or)}$ is completed 'earlier' than the conformational change indicated by $\Delta OD_{280}(II)$. In line with this observation, the chromophore displacement is saturated at a lower field strength than the structural changes associated with $\Delta OD_{280}(II)$ (see fig. 5). Therefore, at least three types of different states are involved in the field-induced structural changes, according to the overall scheme

$$B_0 \rightleftharpoons B_1 \rightleftharpoons B_2 \qquad (23)$$

The rapid phase determined mainly by $B_0 \rightleftharpoons B_1$ (mode I) of the overall structural change is apparent in both wavelength ranges, at 280 and 565 nm. This phase involves concerted orientational changes ($\Delta A_\chi^{(or)}$) and changes in the environment ($\Delta A_\chi^{(ch)}$) of both the retinal and the tryptophan and/or tyrosine residues. The retinal in the protein conformation B_2 has the same orientation and local environment as in the protein conformation B_1 because the retinal displacement saturates in the rapid mode.

The slower changes of structure, rate limited by $B_1 \rightleftharpoons B_2$ (mode II), are clearly visible in the ultraviolet range and as pK changes. The absence of an orientational contribution to phase II indicates that also the tryptophan and/or tyrosine residues are rotationally saturated in phase I, but the local environment of these residues within the protein still varies during phase II; i.e., $\bar{\epsilon}_2 \neq \bar{\epsilon}_1$ at 280 nm.

The sigmoidal onset in the field-on kinetics suggests nucleation-propagation features of cooperative structural transitions [28] (see fig. 1). Cooperativity is also indicated by the relatively large steepness of the transition curves in fig. 5. A quantitative description of the electro-optic data of the purple membrane discs therefore requires a model which comprises multi-step reactions as well as (two-dimensional) cooperativity. A cooperative lattice model and a normal mode analysis in terms of eq. 23 are presented in part II of this series [11].

Here, only the order of magnitude of the reaction dipole moment ΔM is roughly estimated in the framework of a two-state model, using eq. 13 and the slope of the transition curve for $\lambda = 565$ nm in fig. 5. The slope at $\phi^{(ch)} = 0.5$ ($E \approx 1.3 \times 10^5\ V\ m^{-1}$) is given by $(\partial\phi^{(ch)}/\partial E)_{0.5} = [\partial(\Delta X/\Delta X_\infty)/\partial E]_{0.5,565} = 6.7 \times 10^{-6}\ m\ V^{-1}$. Introducing these values into eq. 13 yields the ΔM value at this field strength, if $\theta^0 \ll 1$. At $E \approx 1.3 \times 10^5\ V\ m^{-1}$ we obtain $\Delta M = 6.5 \times 10^{-2}\ C\ m\ mol^{-1}$ at $T = 293$ K.

As discussed in the context of eq. 7, ΔM may be written as

$$\Delta M = N_A(m_1 - m_0) \quad (24)$$

where N_A is Avogadro's constant and m_1 and m_0 the field-parallel dipole moment components of the molecular units involved in the two-state approximation of the structural transition. The evaluation of the ΔM value estimated from eq. 24 yields $m_1 - m_0 = 1.1 \times 10^{-25}$ C m ($= 3.3 \times 10^4$ debye) at $E \approx 1.3 \times 10^5$ V m^{-1}.

Previously, the field strength dependence of the dichroism at 565 nm was analyzed in terms of a restricted orientation of the retinal chromophore, yielding a large polarizability anisotropy of $\Delta\alpha = 2 \times 10^{-30}$ F m^2 (1.8×10^{-14} cm^3), resulting in an induced dipole moment of 2×10^{-25} C m (6×10^4 debye) at $E \approx 10^5$ V m^{-1} per 'cooperative unit' [9].

In any case, the rather large values of the dipole moment differences correspond to the steepnesses of the measured transition curves. However, it is recalled that the numerical values only represent estimates of the order of magnitude. The analysis of $\Delta A_{565}^{(ch)}$ by means of the two-state model instead of a multi-state model includes the assumption that ΔM originates only from the retinal part and its environment. The analysis of $\Delta M_{565}^{(rot)}$ in terms of the restricted orientation model includes the assumption that the chromophore transition moment is fixed to the electric axis [9]. It is doubtful whether these assumptions are very realistic. Furthermore, cooperativity is not explicitly treated. The numerical value of ΔM reflects an apparent reaction quantity; ΔM and thus also the difference $m_1 - m_0$ both contain the cooperative number n_c of molecular dipoles involved in the concerted transition of the cooperative unit:

$$\Delta M = n_c \Delta M^{(intr)} \quad (25)$$

Without independent knowledge of n_c, the intrinsic moment difference $\Delta M^{(intr)}$ (per mol dipole) cannot be evaluated and, vice versa, n_c cannot be determined without knowing $\Delta M^{(intr)}$.

5.4. Electro-optic cycle and photocycle

It was previously mentioned that there are some similarities between the photocycle and the (electro-optic) cycle of structural changes in bacteriorhodopsin, based on a comparison between the electric dichroism and the pH changes in purple membrane suspensions [9,10]. Interestingly, both with respect to the optical density changes in the ultraviolet region as well as to the light scattering changes at 700 nm, the electric field effects show features similar to those of the photo effects. Hess and Kuschmitz [30] suggested that one tryptophan or one or two tyrosine residues change from a non-polar to a polar environment, when bacteriorhodopsins convert to L or M intermediates. Gochev and Christov [31] proposed a light-induced distortion of the retinal followed by a conformational transition of the protein part affecting the native structure of the hydrogen bonds. As we have shown, an external electric field causes a fast concerted change in the environment of both the retinal chromophore and also of the tyrosine and/or tryptophan residues. These fast modes are followed by slower changes involving the tyrosine and/or tryptophan residues and pK shifts of, at least, two proton-binding sites [9,10].

Ort and Parson [32] observed volume changes of the purple membranes upon flash excitation, appearing in two steps. The fast step was interpreted to arise from the release of a proton into the medium; the slow step was attributed to the movement of a proton from one group of the membrane to another proton-binding site. It is thus possible that the electric field-induced light scattering changes observed in our study originate from electrostriction of the purple membranes.

These coincidences and the opposite sign in the sequence of the field-induced pH changes compared to the light-induced pH changes in purple membranes [33] suggest a possible functional role of the membrane electric field [10]. The increase in the membrane potential which results from proton pumping may exert a negative feed-back (reducing proton transport) via an electric field effect directly on the structure of bacteriorhodopsin.

5.5. General conclusions

The large electric polarizability observed in the membrane transport protein bacteriorhodopsin most likely results from an appreciable, yet limited,

displaceability of the ionic groups within this protein. The electric field is apparently able to increase the mean distance between positively and negatively charged amino acid side groups such that the mean dipole moment increases [9]. This electric field effect is suggestive of a possibly general mechanism for electric field-controlled conformational changes in flexible membrane proteins; for instance, those involved in the opening and closing of ion channels in the excitable membranes of nerve and muscle cells or in the (electric) activation of enzyme and receptor proteins.

In particular, some kinetic aspects may gain general functional importance. Compared to the rapid induction of electric field-mediated structural changes in membranes [9,10,15] (see also fig. 4), annealing of the changes after the electric impulse is very slow. The field-induced conformational transitions are thus long-lived compared to the pulse duration; these transitions thus exhibit memory properties. Longevity of field-induced structural changes may also offer a basis for the interpretation of ATP formation by thylakoid membranes after exposure to electric fields [34–36]. When the lifetime of the electric field-induced ATPase activation (to synthesize ATP) is greater than the pulse duration, 'after-field effects' may occur in a way similar to that of the after-field pH changes [10] observed in bacteriorhodopsin of purple membranes. The electric field-induced energization [37] of ATP synthetase would thus be caused by an electric field-induced, long-lived structural change to the active enzyme conformation.

Note added in proof (Received 5th January 1983)

Electric field-induced changes in the intensity of polarized light in purple membrane suspensions were observed by Shinar et al. [38]; a 260 μs time constant characterizing a part of the field-off response was suggested to be possibly due to retinal alignment within the protein.

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