ELECTRIC DICHROISM IN THE PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT Aqueous suspensions of fragments of the purple membrane of *Halobacterium halobium* are exposed to short electric field pulses. The relaxation kinetics of the induced dichroism are studied as a function of environmental factors such as temperature, medium viscosity, and treatment of the membranes with glutaraldehyde and dimethylsulfoxide. The data indicate that the alignment of the retinyl chromophore is due to orientation of the whole membrane fragments with their planes parallel to the electric field, as well as to an intramembrane orientation of bacteriorhodopsin molecules (or of a part of such molecules). Wavelength effects on the dichroic ratio show that weak, out of (membrane) plane components contribute to the chromophore spectrum on the red side ($\lambda > 560$ nm) of the main (α) absorption band as well as in the range of the β band ($\lambda < 480$ nm). The former effect is attributed to exciton interactions, while the latter is assigned to the contribution of a transition to the lowest $^1A_g^+$ state ("*cis*" band). It is also concluded that the transition moment along the short (x) axis, in the plane of the polyene molecule, has a substantial component perpendicular to the membrane plane.

INTRODUCTION

Bacteriorhodopsin (BR), the retinal-protein complex of the purple membrane of *Halobacterium halobium*, functions as an energy converter, transforming light into a transmembrane proton gradient (for a recent review, see reference 1). We have previously shown (2) that the application of short electric field pulses to aqueous suspensions of purple membrane fragments results in transient dichroism phenomena, due to field-induced changes in the orientation of the bacteriorhodopsin molecules. The effect was recently confirmed by Tsuji and Rosenheck (3), who have carried out a theoretical analysis of the induced dichroism. In the present work we report kinetic data related to the nature of the orientation process. The experiments are also extended, covering the visible range of the bacteriorhodopsin absorption spectrum. The effects of wavelength on the field-induced dichroism bear on the electronic transitions and on the orientation of the chromophore in the purple membrane.

EXPERIMENTAL

The preparation of purple membrane fragments from M1 or S9 *H. halobium* and the exposure of their aqueous suspensions to electric field pulses generated in a (Joule heating) temperature-jump instrument (6-70 Messanlagen, GmbH, Gottingen) were previously described (2). Experiments were carried out in which the analyzing light beam was plane-polarized parallel or penpendicular to the applied electric field (~10 kV/cm). The transient changes in absorbance induced by the field pulses (exclusively due to the time-dependent linear dichroism [2]) are denoted by $\Delta A_\parallel$ and $\Delta A_\perp$, respectively. Both parameters were measured as a function of the monitoring light wavelength. Treatment of purple membrane suspensions with the cross-linking reagent glutaraldehyde and with the structure modifier dimethylsulf-

oxide (DMSO) have been previously reported (4,6). A sample of purple membrane, partially regenerated from apo-membranes according to the procedure of reference 14, was supplied by Dr. T. Ebrey, University of Illinois.

RESULTS AND DISCUSSION

The Orientation Process

After the electric field pulse, the dichroism induced in purple membrane fragments exhibits a two-stage relaxation kinetics (2). Half-life values of $\tau^{I}_{1/2} \simeq 300$ μs and $\tau^{II}_{1/2} \simeq 100$ ms were observed for the two processes (I and II) in aqueous purple membrane suspensions at room temperature (2). Fig. 1 shows the combined effects of temperature and of medium viscosity (η), in water-glycerol mixtures, on the values of $\tau^{I}_{1/2}$ and $\tau^{II}_{1/2}$. In the case of the slow relaxation, fair agreement is observed with Perrin's equation (5) $\tau = V\eta/T$, where V is a parameter

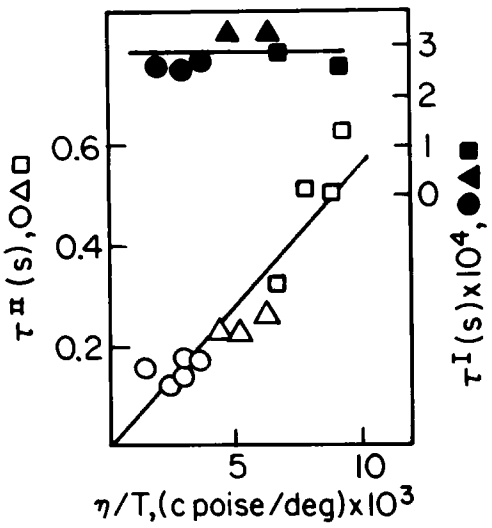

FIGURE 1

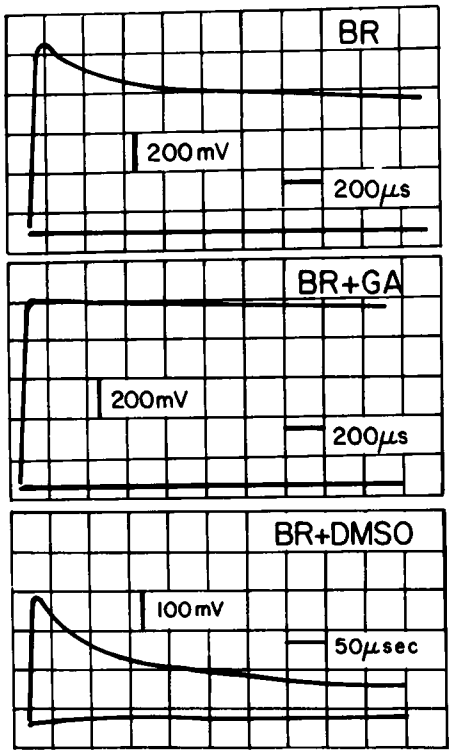

FIGURE 2

FIGURE 1 Effects of temperature and medium viscosity on the half-life values of the fast ($\tau^{I}_{1/2}$) and the slow ($\tau^{II}_{1/2}$) relaxation of the electric field-induced dichroism in purple membrane fragments suspended in water-glycerol mixtures. (●, O, in water; ▲, △, in 20% glycerol-water; ■, □, in 32% glycerol-water).

FIGURE 2 Characteristic traces representing the electric-field-induced dichroism in dark-adapted aqueous solutions of purple membrane fragments. In all cases the absorbance change reported is with a 560-nm monitoring beam polarized parallel to the field (i.e., that corresponding to $\Delta A_{\parallel}$. The light (lower horizontal trace) to dark (not shown) deflection in the absence of the electric field pulse is 4.2 V. (*a*) 12.4 μM BR in water (0.1 NcCl). (*b*) 9 μM BR in 0.025 M phosphate buffer (pH = 6.8) water (0.1 M NaCl) in the presence of 38% DMSO.

depending on the dimensions of the rotating fragment. In contrast, $\tau^{I}_{1/2}$ is essentially independent of η/T. This confirms the hypothesis (2) that process II is due to motion of the whole membrane sheets aligned by the external field, while the fast component (I) should be attributed to an intramembrane chromophore motion unaffected by the viscosity of the external medium.

These conclusions are in keeping with the observation (Fig. 2) that in membrane preparations treated with the cross-linking reagent glutaraldehyde (4), the amplitude of the fast component of the relaxation is essentially eliminated, while that corresponding to process II is unaffected. It is thus evident that cross-linking inhibits the intramembrane orientation process. Moreover, the presence of 38% DMSO, which is known to affect the internal rigidity of the membrane (6), increases the relative amplitude of process I, also reducing $\tau^{I}_{1/2}$ to ~90 μs (Fig. 2). Both phenomena are in keeping with the assignment of the first process to an intramembrane orientation of the chromophore.

The angle α, between the transition moment of the retinyl chromophore, arranged as symmetrical trimers (1), and the purple membrane plane, has been determined by several groups from the linear dichroism of various oriented membrane systems. The values obtained (see discussion in reference 3) are in the range $\alpha = 16° \pm 6°$ (references 7 and 8), $\alpha \leq 24°$ (reference 9), and $\alpha \leq 32°$ (reference 3), indicating that the major component of the chromophore transition moment lies in the plane of the membrane. As previously reported (23), the induced electric dichroism is associated with an absorbance increase when the monitoring beam is polarized parallel to the field ($\Delta A_{\parallel} > 0$, see also Fig. 2) and with an absorbance decrease for perpendicularly polarized light ($\Delta A_{\perp} < 0$). It is therefore evident that the retinyl chromophores have been oriented so that the perpendicular to their transition moments is also perpendicular to the electric field. For chromophores oriented by the alignment of complete fragments, this corresponds to orientation of the fragments with their planes parallel to the electric field. In view of the symmetric two-dimensional (hexagonal-lattice) structure of the purple membrane (10), an electric dipole moment lying in the plane of the membrane should be excluded. Thus, the membrane orientation, parallel to the field, may be achieved only via a dipole moment induced by the external field along the membrane surface. The effect is most probably associated with the polarization of surface charges. Since the relative values of $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are essentially the same for both processes I and II, it appears that the direction of the "internal" chromophore alignment is similar to that associated with the whole membrane fragments; i.e., the external field induces an increase in α by aligning bacteriorhodopsin molecules, or a section of them which carries the retinyl moiety. It is impossible at present to speculate as to the exact nature of this intramembrane motion.

Wavelength Effects on $\Delta A_{\parallel}/\Delta A_{\perp}$

Independently of the specific mechanism by which the BR chromophore is oriented by the external field, an analysis of wavelength effects on the dichroic ratio, $D = \Delta A_{\parallel}/\Delta A_{\perp}$, should bear on the relative polarization of the electronic transitions of BR. In Fig. 3 the dichroic ratio, determined from the values of $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ measured after the completion of the decay process I, is plotted as a function of the wavelength of the monitoring beam. The dichroic ratio, rather than the polarization anisotropy $[(A_{\parallel} - A_{\perp})/(A_{\parallel} + 2A_{\perp})]$, was preferred since it

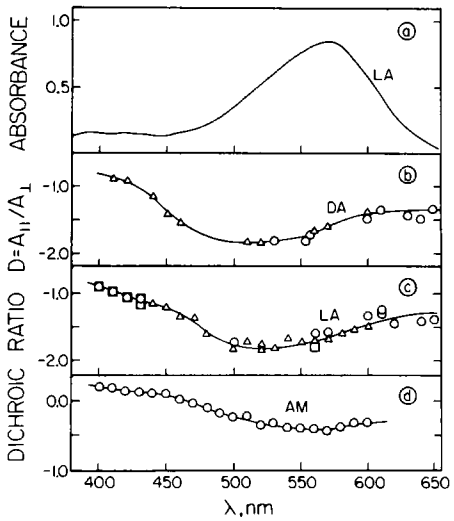

FIGURE 3 Absorption spectrum and wavelength dependence of the transient dichroism induced in BR solutions by an electric field pulse. (*a*) Absorption spectrum of BR_{LA} in water (ϵ_{max} = 63,000 cm^{-1} M^{-1}). (*b*) Dichroism for dark-adapted BR (0.1 M NaCl) M1 (Δ) and S9 (O). Data are recorded 1.8 ms after the pulse. (*c*) Same for light-adapted BR in water (0.1 M NaCl). Different point notations refer to different cultures of M1 (Δ, □) and S9 (O) *H. halobium*. (*d*) Same for 17% partially regenerated (AM) purple membrane (see reference 14).

is free from artifacts which might arise from field-induced changes in the scattering of the solution. It should be pointed out that essentially the same results are obtained for (M1)BR and for (S9)BR which are characterized by different carotenoid contaminations (1). This excludes the possibility that these chromophores may affect the wavelength sensitivity of the dichroism.

It is evident from Fig. 3 that, for either light-adapted bacteriorhodopsin (BR_{LA}, characterized by an all-*trans* chromophore) or dark-adapted bacteriorhodopsin (BR_{DA}, a 1:1 mixture of all-*trans* and 13-*cis* chromophores) (1), the relative contribution of the out of plane component is minimal around 520 nm, increasing both on the red and on the blue. This implies that electronic transitions with a higher out of (membrane) plane intensity contribute to the low energy side of the main (α) band and also in the range between 350 and 480 nm corresponding to the β-band of rhodopsins (for a review, see reference 11).

It is difficult to account for the increased contribution of the out of plane component between 550 and 650 nm in terms of an intrinsic low-energy state of the individual chromophore. The presence of a forbidden transition to a lowest $^1A_g^-$ state on the red side of the intense band due to the main $^1B_u^+$ state, has been confirmed for free retinals in solution (12, 13). However, theoretical calculations for rhodopsins, based on the protonated Schiff base (PRSB) model, indicate that the $^1A_g^-$ state lies above the $^1B_u^+$ state (12, 13, footnote 1). (Estimated values above the ground state for a free PRSB in vacuum are 3.6 eV for $^1A_g^-$ and 2.20 eV for $^1B_u^+$. Moreover, similar to the $^1B_u^+$ state, the $^1A_g^-$ state is theoretically predicted

[1]Dinur, U., B. Honig, and K. Schulten. Manuscript in preparation.

to lead to a transition polarized along the long (y) axis of the retinyl chromophore [2] and thus cannot be responsible for an increase in the out of plane component of the transition moment. On the basis of the hexagonal lattice structure, it is possible to exclude a heterogeneous population of chromophores, i.e., the possibility that the orientation angle α is not identical for all chromophores in the membrane. The most plausible interpretation of the weak variation in the dichroic ratio within the main bands of BR_{LA} and BR_{DA} is in terms of exciton interactions between chromophores in the purple membrane. Experiments were carried out on purple membrane partially (17%) regenerated from chromophore-free apo-membranes. As shown in Fig. 3, only a small variation in $D(\lambda)$ is now observed on the low energy side of the main band. (Note that the value of D is changed relative to the intact membrane, indicating a considerably different chromophore orientation in the modified system). As shown by CD spectroscopy (14), the partially regenerated systems lack the exciton interactions characteristic of the intact membrane (14). Finally, it should be pointed out that the above assignment of the out of plane component on the low energy side of the main bands of BR (DA and LA) calls for a revision of previous exciton models for bacteriorhodopsin which imply that a low intensity, out of (membrane) plane, exciton component contributes to the absorption on the high energy side of the main monomer band (14).

Two electronic states, $^1A_g^-$ (3.6 eV) and $^1A_g^+$ (3.8 eV), are predicted to contribute in the region of the β-band of a free PRSB and, quite similarly, in the case of the chromophore of a pigment such as bacteriorhodopsin (footnote 1, 15). Our data indicate that D increases in the region of the β-bands of BR_{LA} and BR_{DA} (Fig. 3). However, such changes in D are relatively small, implying that the polarization of the β-band is basically similar to that of the main (α) band, i.e., with its major component in the plane of membrane. Such behavior is qualitatively consistent with theoretical calculations which predict that the transition to the $^1A_g^-$ state carries ~ 25% of the intensity associated with the main band and, as in the case of the latter, is polarized mainly along the long (y) axis of the retinal (footnote 2). We suggest that the increase in dichroic ratio in the β-band mainly reflects the superimposed weak transition to a predominantly $^1A_g^+$ state, which is predicted to be polarized along the x axis (footnote 2). Note, however, that the nearby $^1A_g^-$ and $^1A_g^+$ states may be mixed in the pigment by the electrostatic field of the local counterions, so that each of the corresponding transitions may carry some intensity along the polarizaton axis of the other. Accordingly, the "$^1A_g^-$" state may be partially responsible for the increased dichroic ratio in the region of the β-band. Additional arguments are in keeping with locating the "$^1A_g^+$" transition between 350 and 450 nm. First, as calculated by Honig et al. (15), there is a 7000–8000 cm^{-1} energy gap between the lowest $^1B_u^+$ state and the lowest excited $^1A_g^+$ state, both for retinals and protonated Schiff bases, including rhodopsins. This would locate the transition to the $^1A_g^+$ state in *BR* at ~400 nm, in keeping with our present suggestion. Moreover, a vibronic structure observed at low temperatures in the range of the β-bands of both retinals and bacteriorhodopsin was attributed to the $^1A_g^+$ state (15). We thus conclude that $^1A_g^-$ and $^1A_g^+$ states are responsible for the absorption of bacteriorhodopsin in the range from 500 nm down to below 400 nm.

We finally recall that the xy plane corresponds to that of the ideally planar polyene chain

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and that all transitions are fully forbidden along the perpendicular (z) axis. To be consistent with the observed out of (membrane) plane contribution in the 400–500 nm range, it is therefore required that the polyene plane be tilted so as to give the retinal x axis a non zero component perpendicular to the plane of the membrane. Such a distortion for a retinal trimer may be best visualized in terms of a propeller where the blades are distorted with respect to the plane perpendicular to the direction of propagation.

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REFERENCES

1. STOECKENIUS, W., R. H. LOZIER, and R. A. BOGOMOLNI. 1979. Bacteriorhodopsin and the purple membrane of *Halobacteria. Biochim. Biophys. Acta.* **505**:215.
2. SHINAR, R., S. DRUCKMANN, M. OTTOLENGHI, and R. KORENSTEIN. 1977. Electric field effects in bacteriorhodopsin. *Biophys. J.* **19**:1.
3. TSUJI, K., and K. ROSENHECK. 1979. Electric dichroism of purple membrane. *In* Electro-optics and Dielectrics of Macromolecules and Colloids. B. R. Jennings, editor. Plenum Publishing Corp. New York. 77–88.
4. KONISHI, T., and L. PACKER. 1976. Light–dark conformational states in bacteriorhodopsin. *Biochim. Biophys. Res. Commun.* **72**:1437.
5. Sherman, W. V., M. A. SLIFKIN, and S. R. CAPLAN. 1976. Kinetic studies of phototransients in bacteriorhodopsin. *Biochim. Biophys. Acta.* **423**:238.
6. HEYN, M. P., P.-J. BAUER, and N. A. DENCHER. 1975. A natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. *Biochim. Biophys. Res. Commun.* **67**:897.
7. HEYN, M. P., R. J. CHERRY, and U. MULLER. 1977. Transient and linear dichroism studies on bacteriorhodopsin: determination of the orientation of the 568 nm all-*trans* retinal chromophore. *J. Mol. Biol.* **117**:607.
8. BOGOMOLNI, R. A., S. B. HWANG, Y. W. TSENG, G. I. KING, and W. STOECKENIUS. 1977. Orientation of the bacteriorhodopsin transition dipole. *Biophys. J.* **17**:98*a*. (Abstr.)
9. KORENSTEIN, R., and B. HESS. 1978. Immobilization of bacteriorhodopsin and orientation of its transition moment in purple membrane. *FEBS. (Fed. Eur. Biochem. Soc.) Lett.* **89**:15.
10. HENDERSON, R., and P. N. T. UNWIN. 1975. Three dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.).* **257**:28.
11. OTTOLENGHI, M. 1980. The photochemistry of rhodopsins. *In* Advances in Photochemistry. J. N. Pitts, Jr., G. S. Hammond, K. Gollnick, and D. Grossjean, editors. John Wiley & Sons, Inc., New York. 97–200.
12. BIRGE, R. R., J. A. BENNETT, H. L-B. FANG, and G. E. LEROY. 1978. The two-photon spectroscopy of all-*trans* retinal and related polyenes. *In* Advances in Laser Chemistry. A. H. Zewail, editor. Springer Series in Chemical Physics. Vol. 3.Springer-Verlag, Inc., New York. 347–354.
13. BIRGE, R. R., and B. M. PIERCE. 1979. A theoretical analysis of the two-photon properties of linear polyenes and the visual chromophores. *J. Chem. Phys.* **70**:165.
14. EBREY, T. G., B. BECHER, B. MAO, P. KILBRIDE, and B. HONIG. 1977. Exciton interactions and chromophore orientation in the purple membrane. *J. Mol. Biol.* **112**:377.
15. HONIG, B., U. DINUR, R. R. BIRGE, and T. EBREY. 1980. The isomer dependence of oscillator strengths in retinal and related molecules: spectroscopic assignments. *J. Am. Chem. Soc.* **102**:488.