ORIENTATION OF THE CHROMOPHORE PLANE IN PURPLE MEMBRANE

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ABSTRACT Excitation of bacteriorhodopsin (BR) in its β absorption band drives a photocycle identical in the millisecond range, to that excited in the α band of BR. The relative contribution of the two transition dipoles distinguished in the β band to the initiation of the photocycle was established by photoselection experiments. Having this information the orientation of the chromophoric plane was specified by electric dichroism measurements.

INTRODUCTION

Bacteriorhodopsin (BR) is a protein-pigment complex responsible for the light-driven proton pump of Halobacterium halobium (Oesterhelt and Stoeckenius, 1971). The spectrum of BR is characterized by three absorption bands (Becher et al., 1978) in the visible and in the UV range. The α band has an absorption maximum at 568 nm, with an extinction coefficient of $\epsilon \approx 63{,}000$ mol cm. Another extinction region (β band), lacking a clear maximum covers the range between 450 and 300 nm ($\epsilon \approx 13{,}000$ mol^{-1} cm^{-1}). In the UV range, an intense ($\epsilon \approx 75{,}000$ mol^{-1} cm^{-1}) absorption band exists, with an absorption maximum at 280 nm (γ band). While the γ band is attributed to the aromatic amino acids of the protein, the α and β bands are exclusively due to the retinyl chromophore. Light excitation both in the α and in the β band induces practically identical photocycles of the bacteriorhodopsin molecule (Kalisky et al., 1981). Here we present experimental evidence, that excitation of BR in its β band initiates the same photocycle. In order to explore what the contribution of the two transition dipoles distinguished in the β band (Druckmann and Ottolenghi, 1981) is to the photocycle, photoselection experiments were carried out. This method has been used to investigate chromophore motion during the photocycle excited in the band of BR (Czégé et al., 1982). In addition, the orientation of the plane of retinal molecules in purple membrane was specified via electro-optical measurements.

MATERIALS AND METHODS

Purple membrane fragments were prepared from Halobacterium halobium strain R_1M_1 using standard procedures (Oesterhelt and Stoeckenius, 1974). The membranes were suspended in a CH_3COOH KOH buffer, pH = 5.5 or in a 7.5% (wt/wt) polyacrylamide gel incubated in the same buffer. The sample was light-adapted before each measurement and the temperature was maintained at 22°C at all experiments.

A computer controlled flash-photolysis system (Czégé, 1983) was used to obtain data by photoselection measurements. The anisotropy factor (r) was calculated from absorption changes measured by light polarized parallel ($\Delta A''$) and perpendicular ($\Delta A\perp$) to the polarization vector of the actinic light.

$$r = (\Delta A'' - \Delta A\perp)/(\Delta A'' + 2\Delta A\perp). \quad (1)$$

The anisotropy factor is characteristic of the angle between the excited transition dipole nad the measured one (θ). In the case of 1 – 1 transitions:

$$r = 1/5(2 - 3\sin^2\theta), \quad (2)$$

while in the case of 2 excited and 1 measured transition:

$$\Delta A_1'' = C_1\mu_1^2(3 - 2\sin^2\theta_1);\ \Delta A_1\perp = C_1\mu_1^2(1 + \sin^2\theta_1)$$

$$\Delta A_2'' = C_2\mu_2^2(3 - 2\sin^2\theta_2);\ \Delta A_2\perp = C_2\mu_2^2(1 + \sin^2\theta_2), \quad (3)$$

where C_1 and C_2 are the necessary quantum mechanical factors, while μ_1^2 and μ_2^2 are the corresponding oscillatory strengths (Czégé et al., 1982).

Electric dichroism measurements were carried out by ordering the membrane fragments in an alternating electric field. The measuring apparatus was the same as described by Barabás et al. (1983). Absorption changes due to orientation measured with a light polarized parallel ($\Delta A''$) and perpendicular ($\Delta A\perp$) to the electric field strength vector were detected. The reduced dichroisms depend on the electric field strength according to Eqs. 4 and 5. In the case of a single transition:

$$\Delta A\perp/A = (3/2\sin^2\delta - 1)\Phi(\beta, \gamma), \quad (4)$$

and in the case of two transitions:

$$\Delta A\perp = [A_1(3/2\sin^2\delta_1 - 1) + A_2(3/2\sin^2\delta_2 - 1)]\Phi(\beta, \gamma). \quad (5)$$

Here δ, δ_1 and δ_2 are angles between the membrane normal and the transient dipole moment of the chromophore; $\beta = \mu E/kT$; μ, permanent dipole moment; E, electric field strength; k, Boltzmann constant; T, temperature in Kelvin; $\gamma = \alpha E^2/2\,kT$; α, polarizability of the membrane fragments; and Φ is the so-called orientation function (Barbás et al., 1983). Distortions due to light scattering were taken into account by a correction procedure described by Barabás et al. (1983).

RESULTS AND DISCUSSION

Two excited states, $^1A_g^-$ and $^1A_g^+$ are responsible for the absorption of BR in its β band (Druckmann and Otto-

lenghi, 1981). The dipole moment of the transition to $^1A_g^-$ is parallel while that of the transition to $^1A_g^+$ is perpendicular to the polyene chain (Druckmann and Ottolenghi, 1981).

In order to reveal the relative contribution of the two transition dipoles distinguished in the β band to the excitation of the photocycle, experiments were carried out by the photoselection method (see Czégé et al., 1982). The measurements were performed with samples containing purple membranes fixed in polyacrylamide gel, so as to avoid the consequences of the motion of membrane fragments. In a membrane suspension i.e., light scattering effects due to shape changes of bigger ($\phi > 1$ μm) purple membranes correlated to proton extrusion of the membrane strongly influence the anisotropy factor measured by photoselection method (Czégé, 1985). In case of smaller membrane sizes, however, r quickly relaxes to zero due to rotational diffusion of the fragments. Both effects can be avoided by immobilizing the membranes in gel (Czégé et al., 1982). The gel does not influence the spectral and photoelectric properties of bacteriorhodopsin (Dér et al., 1985).

Difference absorption spectra of BR excited in its α and β absorption bands (λ_{ex} = 590 and 337 nm) are compared in Fig. 1. The normalized spectra characteristic to the M intermediate of the BR photocycle are essentially identical. The appearance of the O form was detected by UV light excitation as well (data not shown). These observations suggest that the scheme of the photocycle which the BR undergoes exciting in its β absorption band is, in the millisecond range, the same as that of the normal photocycle.

As a reference, photoselection measurements were performed with excitation in the α band of BR too (Fig. 2, *a* and *b*). The anisotropy factor is constant at both wavelengths (compare Czégé et al., 1982.). For the ground state chromophore r is close to the theoretical value (0.4 if $\theta = 0°$) showing no effect of saturation in exciting the sample, which would diminish the value of r (Czégé et al., 1982). The anisotropy factor characteristic to the M form is also close to 0.4 (Fig. 2*b*), although the isomeric state of the retinal changes from all-*trans* to 13-*cis* during the BR-M transition. The explanation for this is that the r value is not sensitive to small changes in the angle θ at $\theta \approx 0°$, because

$$\left.\frac{dr}{d\theta}\right|_{\theta \to 0} = 0, \text{ according to Eq. (1).} \tag{6}$$

Exciting the BR sample in its β absorption band, r keeps a constant value of $\approx$0.2 at measuring wavelengths both of 560 and 410 nm during the photocycle. The reduced value of r (<0.4) cannot be the consequence of a saturation effect since the corresponding absorption changes are approximately the same in the case of UV and visible light excitation (compare Figs. 2, *a* and *b* and 2, *c* and *d*). It must be concluded therefore, that it is not exclusively the transition to the $^1A_g^-$ or that to the $^1A_g^+$ state which generates photocycle (it would be $r = 0.4$ if $\theta = 0°$ or $r = -0.2$ if $\theta = 90°$), but both of them.

It has been supposed earlier (Druckmann and Ottolenghi, 1981), that the nearby $^1A_g^-$ and $^1A_g^+$ states might be mixed in the pigment by the electrostatic field of local counterions, so that each of the corresponding transitions may carry some intensity along the polarization axis of the other. As we point out below, however, the fact that the value of r is the same for the chromophores of the ground state (at 560 nm) and the M form (at 410 nm) (Figs. 2, *c* and *d*) indicates the existence of pure excited states (namely the $^1A_g^-$ and $^1A_g^+$ states are not combined that way). As a proof let us consider the following arguments. Knowing that $\theta_1 = 0°$ and $\theta_2 = 90°$, similarly to Eq. (6), one can derive the following derivatives according to Eqs. (1) and (3):

$$\left.\frac{\partial r}{\partial \theta_1}\right|_{\theta_1 \to 0°} = 0$$

$$\left.\frac{\partial r}{\partial \theta_2}\right|_{\theta_2 \to 90°} = 0, \tag{7}$$

that is in the case of pure $^1A_g^-$ and $^1A_g^+$ states r is insensitive to small changes of θ_1 and θ_2, like the isomerisation of the retinal is expected to cause. On the basis of Eqs. (1) and (3) it is easy to see, that mixing of the $^1A_g^-$ and $^1A_g^+$ states would result in different r values for the ground state and the M form.

Accepting the existence of pure excited states, the experimental results can be quantitatively interpreted. Using the abbreviations $a = \frac{1}{5}(\Delta A_1'' + 2\Delta A_1\perp)$ and $b = \frac{1}{5}(\Delta A_2'' + 2\Delta A_2\perp)$, it follows from Eqs. (1) and (3), with the values of $\theta_1 = 0°$ and $\theta_2 = 90°$, that

$$r = (2a - b)/5(a + b). \tag{8}$$

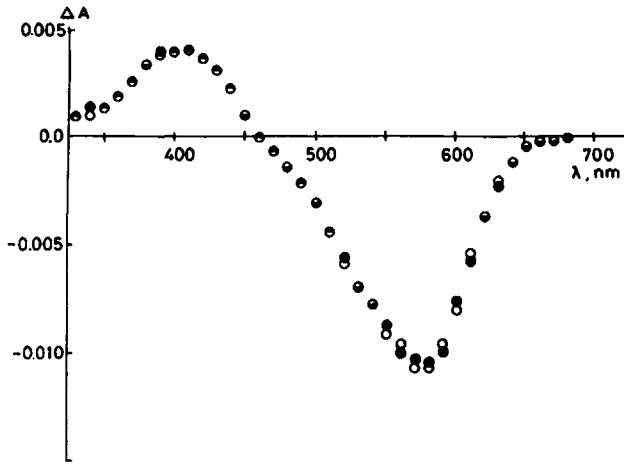

FIGURE 1. Normalized difference absorption spectra of BR excited in its α (O) and β (●) absorption band, 200 μs after the flash.

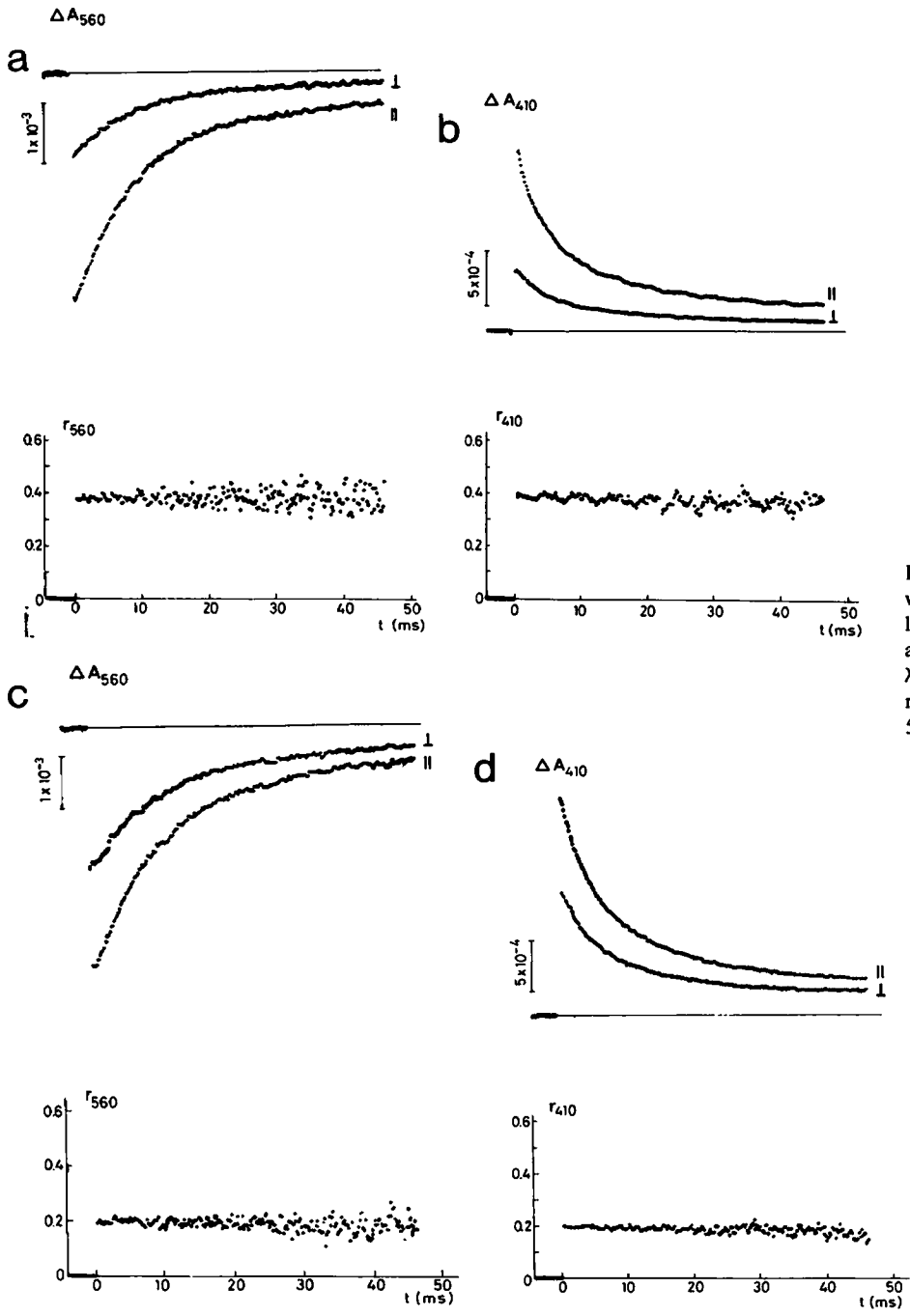

FIGURE 2. Absorption changes measured with light polarized parallel and perpendicular to the polarization of the exciting light, and the corresponding anisotropy factors. (*a*) λ_{ex} = 590 nm, λ_{meas} = 560 nm; (*b*) λ_{ex} = 590 nm, λ_{meas} = 410 nm; (*c*) λ_{ex} = 337 nm, λ_{meas} = 560 nm; (*d*) λ_{ex} = 337 nm, λ_{meas} = 410 nm.

The measurements yielded $r = 0.195 \pm 0.005$, thus $a/b = 1.93 \pm 0.007$ follows from Eq. (8). The physical meaning of this ratio turns out considering Eq. (3):

$$a/b = C_1\mu_1^2/C_2\mu_2^2, \tag{9}$$

where C_1 and C_2 are quantum mechanical factors proportional to the quantum efficiencies, while μ_1 and μ_2 are the absolute values of the transition dipole moments corresponding to $^1A_g^-$ and $^1A_g^+$, respectively.

Although there are no data available in the literature for the quantum efficiencies in the β band of bacteriorhodopsin, in the case of bovine rhodopsin, however, the same quantum yield was found for bleaching in the β band as in the main band (M., Ottolenghi, 1980). It seems reasonable, therefore, to consider this possiblity in the case of bacteriorhodopsin as well.

Provided that the quantum efficiences corresponding to the two excited states in the β band are the same ($C_1 = C_2$) as the quantum efficiency in the α band, from Eq. (9) we obtain 1.93 ± 0.007 for the ratio of the oscillatory strengths (i.e., for that of the absorption coefficients).

In this special case, further information can be gained about the orientation of the retinal molecule by linear dichroism measurements. The membrane fragments can

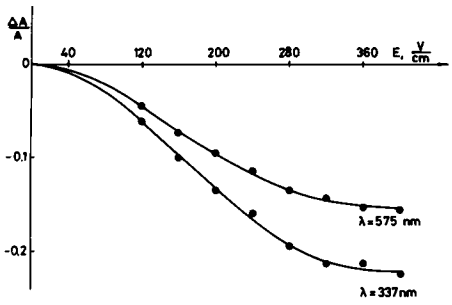

FIGURE 3. Relative absorption changes measured by light polarized perpendicular to the electric field ($\Delta A\perp/A$). λ_{meas} = 575 and 337 nm. $A_{575} = 0.39$; $A_{337} = 0.09 \pm 0.01$.

be ordered in an electric field (Keszthelyi, 1980). The reduced dichroisms ($\Delta A''/A$ and $\Delta A\perp/A$) are the function of the electric field strength and the angle between the membrane normal and the transition dipole moment of the chromophore, according to Eqs. (4) and (5). The reduced dichroisms ($\Delta A\perp/A$) measured at 560 and 337 nm are depicted in Fig. 3. Since for high alternating electric fields $\Phi(\beta, \gamma) \rightarrow -\frac{1}{2}$ (Barabaś et al., 1983), δ, δ_1, and δ_2 can be expressed by Eqs. (4) and (5), using the saturation values of the reduced dichroisms. At 560 nm a single transition dipole can describe the absorption, while at 337 nm both the transitions to the $^1A_g^-$ and $^1A_g^+$ states should be considered. Since the transition to the $^1A_g^-$ state is parallel to the transition dipole in the α band $\delta = \delta_1$ follows. Taking into account our previous assumption $/A_1 = (1.93 \pm 0.07)A_2/$, a calculation based on Eqs. (4) and (5) yields the following values: $\delta_1 = (69.6 \pm 1.0)°$ (see also Barabás et al., 1983) and $\sin \delta_2 = 1.03 \pm 0.03$, from which $\delta_2 = 90°$ was concluded. This would mean that the transition to the $^1A_g^+$ excited state is in the plane of the membrane.

The transitions $^1A_g^-$ and $^1A_g^+$ are both in the polyene plane (Druckmann and Ottolenghi, 1981) and perpendicular to each other, consequently the angle between the plane of the retinal and that of the membrane should be equal to 20°.

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