IMMOBILIZATION OF BACTERIORHODOPSIN AND ORIENTATION OF ITS TRANSITION MOMENT IN PURPLE MEMBRANE

Rafi KORENSTEIN and Benno HESS
Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddamm 201, 4600 Dortmund, FRG

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1. Introduction

Flash photoselection studies of rotational mobility of bacteriorhodopsin (BR) within the purple membrane matrix have been confined to the ms time range and showed that either the protein is completely immobilized or that its rotational relaxation time is slower than 20 ms [1,2]. Recently a rotational rate constant of 20 s^{-1} was observed which was attributed to the mobility of the retinal chromophore [3].

So far the flash photoselection experiments with suspended purple membrane fragments in water have encountered two difficulties:

(i) The life time of the longest lived '412' intermediate of bacteriorhodopsins photocycle limits the detection of rotational diffusion relaxation times only down to 20 ms.

(ii) The rotation of the whole purple membrane fragment (diameter of ~0.5 μm) in water introduces an additional mode for the decay of the transient dichroism, which complicates the analysis of the time-dependent dichroism, especially when the size population of the purple membrane fragments is heterogeneous. In order to prevent the whole fragments from rotating, they were incorporated into a polymer [3] or whole envelope cells were used [4].

Linear dichroism refers to differential absorption of plane polarized light by an oriented sample. There are various methods of preparing oriented samples [5], some of which have been applied to orient purple membrane fragments:

(a) Spreading of the purple membrane fragments on a glass slide and consequent formation of thin layers after drying [6,7].

(b) Orientation in molecular films in an air–water interface [8].

(c) Orientation by magnetic [9] or electric fields [10,11].

We have chosen method (a) to obtain oriented preparations of purple membrane, since it has been been found from X-ray diffraction measurements [6,7] that the purple membrane fragments orient themselves when dried on a glass slide with their membrane planes parallel to the glass surface.

We present here results on transient dichroism in the thermal isomerization of all-*trans*→13-*cis* retinal and on the linear dichroism in the absorption of bacteriorhodopsin. The results show that bacteriorhodopsin (as monomer, trimer or even as cluster of trimers) is immobilized within the native purple membrane matrix and that the retinal chromophore forms an angle $\leqslant 27°$ with the plane of the membrane.

2. Materials and methods

Purple membrane was isolated from *Halobacterium halobium* NRL R_1M_1 [12]. Thin purple-membrane layers were prepared by drying concentrated suspensions of purple membrane in water (pH 7.2) on a glass slide. Samples of A_{570} 1.2–1.6 were prepared for the BR^{LA}–BR^{DA} photoselection experiments (in light-adapted bacteriorhodopsin, BR^{LA}, and in dark adapted bacteriorhodopsin, BR^{DA}, the retinal composition is all-*trans* and 13-*cis*:all-*trans*, 1:1,

correspondingly [12]). In linear dichroic measurements much thinner layers with A_{570} 0.0132–0.0066 were used. The average thickness of the thin purple-membrane layer was determined by scanning electron microscopy. The sample for the photoselection studies was inserted in a cuvette and then equilibrated with 94% relative humidity for 24 h. The glass slides used in linear dichroism were only half covered with thin purple-membrane layer, the uncovered half serving as the reference in the measurement. Linear dichroic measurements were performed with purple membrane layers equilibrated with the room humidity (45% relative humidity).

The schematic orientation of the glass slide relative to the measuring light axes, in the linear dichroic studies is shown in fig.2a. The parallel light axes are defined relative to the long axis of the glass slide. The experimental set up for measuring linear dichroism is described in [13].

The BR^{LA}–BR^{DA} photoselection measurements were carried out by irradiating dark-adapted thin purple-membrane layer with light > 500 nm through two 'Polarex' linear polarization filters. The irradiation time period was chosen so as to provide only 50% conversion of BR^{DA} into BR^{LA} in order to eliminate reduction in the photoselection effects under saturating light conditions. The photoselection, under saturating light conditions, is no longer from a random sample, since the ground state is significantly and anisotropically bleached [14]. The BR^{LA}–BR^{DA} photoselection experiments were carried out at two geometries:

(i) The preparation was excited by linearly polarized light with an axis normal to the plane of the glass slide, then the glass slide was rotated by 90° and analyzed with polarized light with axes normal to the glass-slide plane.

(ii) Both excitation and analysis light axes formed simultaneously an angle of 45° with the plane of the glass slide.

The $BR^{LA} \rightarrow BR^{DA}$ transition was recorded with a single beam spectrophotometer. In the experimental set up of this single beam spectrophotometer the measuring light of 400 W W-I_2 lamp passed through a Bausch and Lomb monochromator (no. 33-86-02), was focussed onto the thin layer preparation and then passed an interference filter to the photomultiplier (EMI 9634 QR). The amplified transient output current of the multiplier was passed through a variable RC filter to a digital scope (Nicolet, model 1090), where the filter setting provided filter relaxation time equal to 0.05% of the oscilloscope total time-sweep width. The oscilloscope trace was recorded on a Hewlett Packard $X-Y$ recorder. Direct transient changes in absorbance were obtained by connecting the output of the amplifier to a log ratio module. The $BR^{LA} \rightarrow BR^{DA}$ decay process was recorded at 590 nm, at which the BR^{LA}–BR^{DA} absorption difference spectrum is maximal.

The anisotropy factor $r(t)$ is defined as

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)}$$

where $A_{\parallel}$ and $A_{\perp}$ are ΔA_{590} values, with the polarized light parallel and perpendicular to the exciting polarized light, respectively.

3. Results and discussion

3.1. *Rotational immobilization of bacteriorhodopsin*

Photoselection studies of the dichroic relaxation in bacteriorhodopsin were performed with thin purple-membrane layers equilibrated with 94% relative humidity. Under such conditions bacteriorhodopsin undergoes a complete photocycle and has the same spectroscopic and kinetic characteristics found for suspended purple membrane in water [15]. In order to detect very slow rotations of bacteriorhodopsin within the purple matrix one has to use a photo-transient of slower relaxation time than available by the '412' intermediate of the photocycle and to immobilize the purple membrane fragments. Thus we have chosen to measure the transient dichroism of the long $BR^{LA} \rightarrow BR^{DA}$ thermal transition (relaxation time constant of 14 min at 40°C), which corresponds to an all-*trans*→13-*cis* isomerization of the retinal chromophore. Employing thin purple-membrane layer on a glass slide, the purple membrane fragments were completely immobilized to the glass surface.

A transient dichroism in the decay kinetics of $BR^{LA} \rightarrow BR^{DA}$ process is shown in fig.1a. The spectral changes at 590 nm are only due to one component (all-*trans*), since the photocycle spectral changes in the same spectral region take place in the ms time domain. Thus the anisotropy factor can be con-

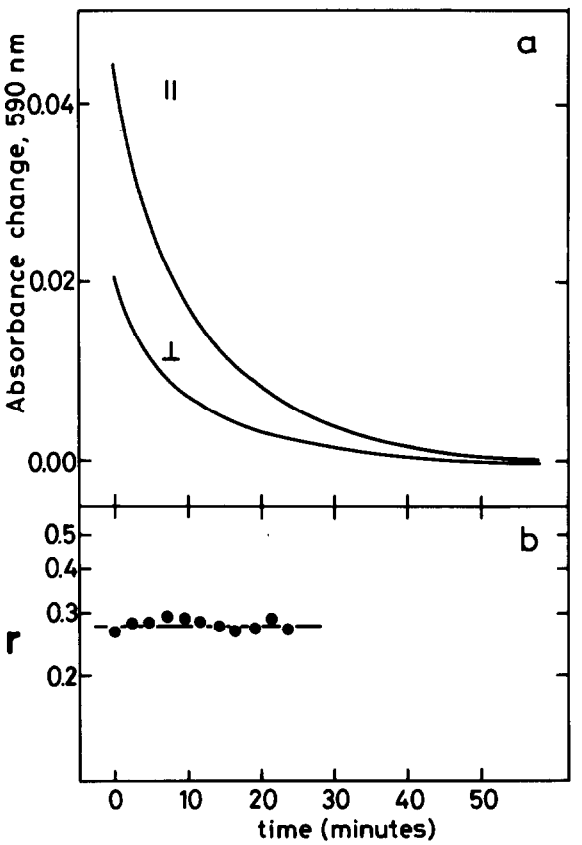


Fig.1a. Transient dichroism in the decay kinetics of $BR^{LA} \rightarrow BR^{DA}$ measured in a thin layer of purple membrane (equilibrated with 94% relative humidity) at 40°C. The axis of both the exciting and the analysing light formed an angle of 90° with the glass slide plane. Fig.1b. Time dependence of the anisotropy parameter $r(t)$ as calculated from (a).

sidered as independent of the $BR^{LA} \rightarrow BR^{DA}$ relaxation time [21]. Analysis of the data shows that the anisotropy factor $r(t)$ remains constant over time range of 25 min (fig.1b). The transient dichroic measurements were performed at 40°C, after the purple membrane had undergone thermal transition in the 23–30°C temp. range [16]. Thus even under such conditions of reduced microviscosity no rotational freedom can be detected. A higher anisotropy factor (r=0.3) observed when the exciting polarized light formed an angle of 45° with the glass slide plane as compared to the value obtained when the exciting light is perpendicular to the glass slide plane (r=0.27), results from linear dichroic effects arising from the orientation of the purple membrane fragments with their planes parallel to the plane of the slide glass. Moreover the value of 0.27 obtained for $r(t)$, is lower than the maximum theoretical value of 0.4, due to instrumental factors. Recently a linear dichroic effect in the circular dichroism of aggregated purple-membrane fragments in solution has been observed [17].

It can be concluded that bacteriorhodopsin is completely immobilized within the purple membrane matrix. The results eliminate the possibility for rotational freedom of bacteriorhodopsin monomer, trimer or even a big cluster of trimers. The results also exclude the possibility for the chromophore to rotate about those axes which would lead to a dichroic decay, suggesting a strong retinal–opsin interaction. Using fluorescence depolarization, the microviscosity of the lipid domains in the purple membrane was found to be 5 poise [16]. Since the present study shows that bacteriorhodopsin is immobilized within the purple membrane matrix, it is clear that the microviscosity that bacteriorhodopsin experiences is mainly due to protein–protein interactions and not due to lipid–protein interactions.

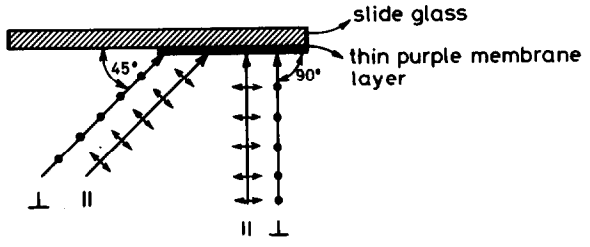
3.2. *Linear dichroism of bacteriorhodopsin*

Linear dichroic measurements of bacteriorhodopsin in thin purple-membrane layers were performed at two geometries, as shown in fig.2a. No dichroism was observed when the polarized light axes were perpendicular to the plane of the membrane (90°, || and 90°, ⊥). This would be expected if the chromophores are randomly oriented about the axis normal to the plane of the glass slide. However a dichroism was observed (fig.2b) when the light axes formed an angle of 45° with the plane of the glass slide (45°, || and 45°, ⊥). The angle between the transition moment of the chromophore and the plane of the membrane can be derived from the measured dichroic ratio. The dichroic ratio for chromophores randomly oriented about the normal to the plane of the membrane is given by [18]:

$$D = \sin^2\alpha + 2\tan^2\phi\cos^2\alpha \qquad (1)$$

where ϕ is the angle between the transition moment of the chromophore and the plane of the membrane,

Fig.2a



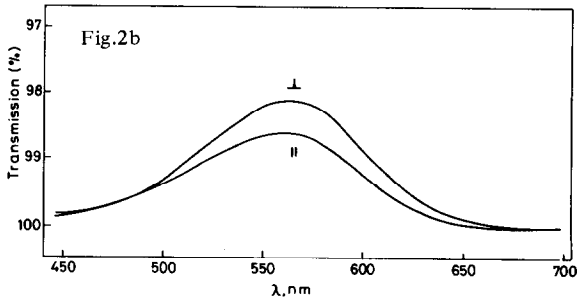


Fig.2a. Scheme of the sample and the polarized light used in the linear dichroic absorption measurement. The glass slide was only half covered, the uncoated part serving as a reference. The purple membrane fragments were oriented with their membrane planes parallel to the glass slide plane. The parallel (∥) and the perpendicular (⊥) orientations of the polarized light were defined parallel to the long and the short axes of the rectangular glass slide. Fig.2b. Linear dichroic spectrum of thin purple membrane layer (equilibrated with a relative humidity of 45%) measured with polarized light axes forming an angle of 45° with the glass slide plane.

α is the angle of the polarized light axis with the plane of the membrane and D is the dichroic ratio. Correcting for the index of refraction, n, using Snell's law we obtain

$$D = 1 - \frac{\cos^2\alpha}{n^2} + 2\tan^2\phi \cdot \frac{\cos^2\alpha}{n^2} \qquad (2)$$

However in order to account for the existence of multiple reflections in thin films a correction in the analysis of the dichroic data should be introduced. It can be shown that if the film thickness is larger compared to the wavelength of the analysing light, the effect of multiple reflections can be neglected. However when the thickness of the film is equal or smaller than the wavelength of light, the dichroic ratio will depend strongly on the film thickness [19]. Therefore we measured the dichroic ratio in purple membrane samples of different thicknesses (d). The results are: $D = 0.89$ ($A = 0.6, d \sim 4\ \mu m$); $D = 0.85$ ($A = 0.24, d \sim 1.3\ \mu m$); $D = 0.78$ ($A = 0.015, d < 1\ \mu m$). When substituting the various D values in (2), where $\alpha = 45°$ and $n = 1.5$ [20], we obtain values of 26.7°, 21.9° and 4° for ϕ, correspondingly. Thus it can be stated that $\phi \leqslant 27°$. A value of 23.5° was measured in molecular films in an air water interface [8], whereas a value of 19° resulted from measurements in thin purple-membrane layers [21]. The values differ due to disorder in the alignment of the purple membrane fragments as well as to multiple reflection effects, where the first cause predominates in thick layers while the second does in thin layers.

Another complication arises from the fact that the retinal composition (all-*trans*/13-*cis*) of the light-adapted bacteriorhodopsin (BR^{LA}) is dependent on the state of hydration [22]. It was shown that only at the highest hydration state (94% relative humidity) is the retinal configuration all-*trans*. However under the experimental conditions of 45% relative humidity the retinal composition of BR^{LA} is 70% all-*trans* and 30% 13-*cis* [22]. Thus any study of linear dichroism under conditions where the highest relative humidity is not maintained, will meet with the problem of interpreting linear dichroism of a mixture of 13-*cis* and all-*trans* retinal.

The finding that the angle between the transition moment of the chromophore and the plane of the membrane is $\leqslant 27°$ raises the question whether such an angle would affect the efficiency of light absorption in the intact bacteria. *Halobacterium halobium* is a cylindrical cell, where the purple membrane forms special domains in the cell membrane. Since the absorption of the light is a scalar product between the electric vector of the light and the transition moment of the chromophore, the maximal absorption would be obtained when the light axis is perpendicular to the transition moment of the chromophore (we can assume random orientation of the transition moments of the chromophores about the normal axis to the membrane plane). However since the sun-light illuminates, with a parallel light, only a half cylinder of the bacteria, the light axis of the light will form different angles with the transition moment

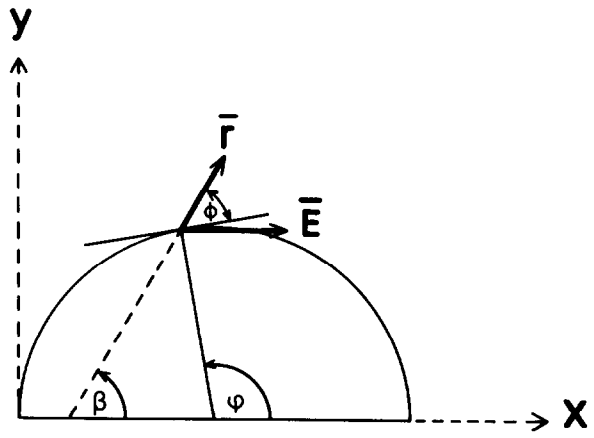


Fig.3. Scheme for the interaction of the electric vector of the light with the transition moment of the retinal chromophore over a half circle. $\bar{r}$ is a unit vector of the transition moment of the chromophore. The electric vector of the light, $\bar{E}$, is defined in the x-direction (light axis is in the y-direction). ϕ is the angle between the transition moment of the chromophore and the plane of the membrane and β is the angle between $\bar{r}$ and $\bar{E}$.

of each chromophore, depending on the localization of the chromophore on the half circle of the cylindric cross section. The absorption process is proportional to the scalar multiplication of the electric vector $\bar{E}$ with the transition moment vector $\bar{r}$. This interaction can be reduced to a two dimensional representation as shown in fig.3. In order to obtain maximal absorption, the sum of the transition moment components in the x-direction (r_x) over the half circle should be maximal. The absolute value of the sum of the transition moment vector unit components in the x direction, $|R_x|$, is given by:

$$|R_x| = {}_0\int^{180} \cos\beta \, d\varphi|$$

$$= |{}_0\int^{180} \cos(\varphi - 90 + \phi)\, d\varphi|$$

$$= 2$$

Thus the absorption efficiency is independent of the angle between the chromophore and the membrane plane, when the protein is distributed continuously on the surface of a cylinder.

4. Conclusions

The complete immobilization of bacteriorhodopsin even under conditions of reduced microviscosity reflects the exceedingly strong protein–protein interactions within the purple membrane matrix. These strong interprotein forces should play a key role in any cooperative transition that the bacteriorhodopsin undergoes. Thus, such forces can introduce cooperativity into the kinetics of the photocycle of bacteriorhodopsin or even into the proton-transfer processes. The method employed in the photoselection study can be extended to other systems where very slow motions are to be expected.

The transition moment of the retinal chromophore forms an angle smaller than 27° with the plane of the purple membrane. The efficiency of light absorption by *Halobacterium halobium* is independent of the angle between the transition moment of the retinal chromophore and the plane of the membrane when assuming the shape of the bacteria to be that of a cylinder. However, a deviation of the circular cross section towards an elipsoidic one will introduce an angular dependence in the light absorption process.

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References

[1] Razi Naqvi, K., Gonzales-Rodriques, J., Cherry, R. J. and Chapman, D. (1973) Nature New Biol. 245, 249–251.
[2] Sherman, W. V., Slifkin, M. A. and Caplan, S. R. (1976) Biochim. Biophys. Acta 423, 238–248.
[3] Sherman, W. V. and Caplan, S. R. (1977) Nature 265, 273–274.
[4] Cherry, R. J., Heyn, M. P. and Oesterhelt, D. (1977) FEBS Lett. 78, 25–30.
[5] Hofrichter, J. and Eaton, W. A. (1976) Ann. Rev. Biophys. Bioengin. 5, 511–561.
[6] Blaurock, A. E. and Stockenius, W. (1971) Nature New Biol. 233, 152–155.
[7] Blaurock, A. E. (1975) J. Mol. Biol. 93, 139–158.
[8] Bogomolni, R. A., Hwang, S. B., Tseng, Y. W., King, G. I. and Stoeckenius, W. (1977) Biophys. J. 17, 98a (abstr. W-POS-C13).

[9] Neugebauer, D. Ch., Blaurock, A. E. and Worcester, D. L. (1977) FEBS Lett. 78, 31–35.
[10] Shinar, R., Druckmann, S., Ottolenghi, M. and Korenstein, R. (1977) R. Biophys. J. 19, 1–5.
[11] Eisenbach, M., Weissmann, C., Tanny, G. and Caplan, S. R. (1977) FEBS Lett. 81, 77–80.
[12] Oesterhelt, D., Meentzen, M. and Schumann, L. (1973) Eur. J. Biochem. 40, 453–463.
[13] Möbius, D. and Bücher, H. (1972) in: Physical Methods of Chemistry (Weissberger, A. and Rossiter, B. W. eds) part III, pp. 675–678, Wiley-Interscience, NY.
[14] Albrecht, A. C. (1970) Prog. React. Kinet. 5, 302–334.
[15] Korenstein, R. and Hess, B. (1977) Nature 270, 184–186.
[16] Korenstein, R., Shermann, W. V. and Caplan, S. R. (1976) Biophys. Struct. Mechanism 2, 267–276.
[17] Reed, T., Hess, B. and Doster, W. (1978) Biochim. Biophys. Acta in press.
[18] Cherry, R. J., Hsu Kwan and Chapman, D. (1972) Biochim. Biophys. Acta 267, 512–522.
[19] Engelsen, den D. (1972) J. Phys. Chem. 76, 3390–3397.
[20] Brith-Lindner, M. and Rosenheck, K. (1977) FEBS Lett. 76, 41–44.
[21] Heyn, M. P., Cherry, R. J. and Müller, U. (1977) J. Mol. Biol. 117, 607–620.
[22] Korenstein, R. and Hess, B. (1977) FEBS Lett. 82, 7–11.