Biochimica et Biophysica Acta, 592 (1980) 258—266

BBA 47901

ELECTRIC FIELD PROMOTION OF THE BACTERIORHODOPSIN BR_{570} TO BR_{412} PHOTOCONVERSION IN FILMS OF *HALOBACTERIUM HALOBIUM* PURPLE MEMBRANES

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(Received October 1st, 1979)
(Revised manuscript received March 13th, 1980)

Key words: Bacteriorhodopsin; Intermediate BR_{412}; Photoconversion; Dielectric polarization; Purple membrane (Halobacterium halobium)

Summary

The combined action of electric field (10^5—10^7 V · m^{-1}) and light (380—580 nm, 80 W · m^{-2}) activating the photoenergetic reaction of bacteriorhodopsin (BR) in dry films of purple membranes from *Halobacterium halobium* was studied. A new stimulating effect of the field on the BR_{412} intermediate accumulation in the normal photochromic cycle of BR_{570} has been observed. The formation of the product BR_{412} is supposed to be accompanied by specific rearrangements of certain charged, polar and polarizable groups in the BR pigment-protein matrix. Such an intrinsic polarization could be promoted by an external electric field, the displacement vector of those groups being oriented in the direction of the field. The dielectric polarization properties of the purple membranes have been demonstrated by electret-thermal analysis.

Introduction

In previous works [1—3] on dry films of BR-containing purple membranes from *Halobacterium halobium* R_1, we have observed and described in detail the bathochromic shift of the main absorption band of the light-adapted BR_{570} induced by an external electric field (10^6—10^7 V · m^{-1}). The spectrum of the bathoform thus produced was found to be similar to that of the initial photointermediate, K, in the photochromic cycle of BR_{570} [4,5].

Abbreviations: BR, bacteriorhodopsin; ΔA_λ, absorbance change with the subscript indicating the wavelength of the differential maximum.

Theoretical calculations of electrically-induced spectral changes were also made, using the method of Mathies and Stryer [17].

Electrically-induced changes of the absorption, ΔA, are defined by the expression [6]:

$$\Delta A = A\left[a + b\frac{(\partial/\partial\nu)(A/\nu)}{A/\nu} + c\frac{(\partial^2/\partial\nu^2)(A/\nu)}{A/\nu}\right]$$

The calculations were made on the assumption that the electric field had no effect on the shape of the spectrum. a, b and c are coefficients depending upon both the free dipole moment and the polarizability of the pigment molecule and the electric field in its vicinity. It means they are the functions of the external electric field and polarization properties of the medium.

The electrically-induced difference spectra (both experimental and calculated, see Fig. 2, curves 2, 3, respectively) are rather close. The electrically-induced signal of the bathoform at 630 nm (ΔA_{630}) appeared to be quadratic in field strength [2].

The formation of the batho-BR in the normal BR_{570} photocycle is, evidently, accompanied by the processes of dielectric polarization in the vicinity of the electronically-excited retinal. The latter can, evidently, be reproduced by electric exposure in model experiments in dry films. This polarization may include displacements of the protons near the chromophore group as the first step of their translocation (the specific function of BR). BR is supposed to be able to transmit electrically-induced conformational changes from the nearest retinal surrounding to other parts of this macromolecular complex, and vice versa.

Pronounced structural rearrangements of BR in response to pulsed electric exposure ($2 \cdot 10^4\ V \cdot m^{-1}$) have recently been demonstrated in the laboratory of Hess: in a suspension of native purple membranes and their apofragments, the field-induced absorbance changes at 280 nm in proteins were observed to rise within microseconds [7].

In the present work we investigated the combined action on BR of electric field and blue-green light, activating the photoenergetic reaction. A new phenomenon has been revealed — the stimulation of photoconversion of the BR_{570} to a BR_{412} intermediate by a constant electric field. From the data it is concluded that the formation of the BR_{412} may include specific rearrangements of certain charged, polar and polarizable groups in the BR pigment-protein matrix. Such an intrinsic polarization could be probably promoted by an external electric field, if the displacement vector of those groups be aligned in the field direction. The dielectric polarization properties of the purple membranes have been demonstrated by electret-thermal analysis of the preparations.

Materials and Methods

The fraction of purple membranes from *Halobacterium halobium*, strain R_1, was provided by the Bioenergetics Department of the A.N. Belozersky Laboratory at Moscow State University.

A semi-transparent aluminum film (50—80 Å thick, 60—70% transparency) evaporated in vacuum on the surface of a glass plate served as one of the capacitor plates.

Purple membrane films were prepared by vacuum drying of a drop of the suspension put on the aluminum electrode at room temperature. The second translucent aluminum electrode was evaporated in vacuum onto the upper surface of the dry preparation. The electric resistance of films was in the range 10^7—10^9 Ω, their electrical capacity was (2—8) · 10^{-11} F.

In calculating the thickness of a membrane film we used the plane capacitor formula. The thickness thus calculated was (2—8) · 10^{-6} m. Thickness control measurements were made by direct micrography of a broken glass plate with a film. Control measurements gave similar values.

Prior to experiments samples were allowed 24 h for equilibration with the room humidity (65—70%).

The procedure was previously described in Refs. 13 and 26.

The films thus prepared had the absorption maximum at about 562 nm which is characteristic of the dark-adapted BR. Before measurements the films were irradiated for 10 min with blue-green light, $\lambda < 600$ nm, at room temperature (20°C); this resulted in a slight increase in sample extinction coefficient and the absorption maximum shift to 568 nm (the light-adapted BR), in good agreement with the observations of [8].

It is worth mentioning that the spectral characteristics of the electrically-induced bathoform were independent of whether the samples were light-adapted or not.

The field effect on photo-induced BR_{412} was studied, using a steady-state blue-green (380—580 nm) background.

In agreement with the results reported in [5,9] we have also observed the whole cycle of photochromic conversions of BR_{570} in air-dried films of purple membranes. However, due to dehydration some steps of the photocycle occurred on a much longer time scale. Thus, in our films the decay time ($t_{1/2}$) of flash-induced state BR_{412} is about 50—60 ms, while in an aqueous suspension it is only 3—4 ms. Because of a slow relaxation of BR_{412} it was possible to investigate the kinetics and spectral characteristics of this intermediate using a conventional single-beam differential spectrophotometer [10]. Mechanical modulation of actinic and measuring beams in the instrument provides a means for time separation between excitation and measurement (dark interval, 7 ms).

It is known that drying without any additional orienting procedures aligns the purple membranes in a film parallel to the plane of the glass plate [11]. Taking into account the geometric shape of the purple membrane (flat discs of 5000 Å diameter and 50 Å thickness [12]) it is not unreasonable to assume that a dry film may include oppositely oriented bulks of purple membranes [2].

For low temperature experiments, the sample was attached to a metal holder of an optical cryostat fitted in a cuvette compartment of the spectrophotometer [10].

Electret experiments were carried out as described in Refs 13 and 14 using the same cryostat with a controlled heating element. The latter provided a means for the sample (pre-cooled to −160°C in the electric field, 2 · 10^7 V · m^{-1}) to be warmed gradually at a rate of 7 K/min. Sample temperature was monitored by means of a thermocouple held in contact with the sample. Some other experimental details are given in the text and the legends to figures.

Results and Discussion

Simultaneous exposure of purple membrane films to electric field and blue-green irradiation

The following series of experiments was carried out:

(1) The absorbance changes of the sample induced by an external electric field ($2 \cdot 10^7$ V $\cdot$ m^{-1}) were measured in the dark;

(2) The sample was illuminated with blue-green light (380—580 nm, 80 W $\cdot$ m^{-2}) activating the photoconversion of BR_{570} and electric field effects were then studied on the background of this irradiation;

(3) The sample was exposed first to the field and then to blue-green light and the resultant effects were registered.

Fig. 1 represents typical kinetics of absorbance changes of BR_{570} at 570 nm (ΔA_{570}), 630 nm (ΔA_{630}) and 420 nm (ΔA_{420}) in purple membrane films for all the three experiments.

Along with the effects reported earlier (an electrically induced BR bathochromic shift [1,2]) a new phenomenon — a field stimulation of BR_{412} accumulation in the normal photocycle of BR_{570} — has been observed. In fact, in the dark the electric field had no effect on the absorption at 420 nm (Fig. 1, version 1). But the electric field applied under continuous blue-green illumination

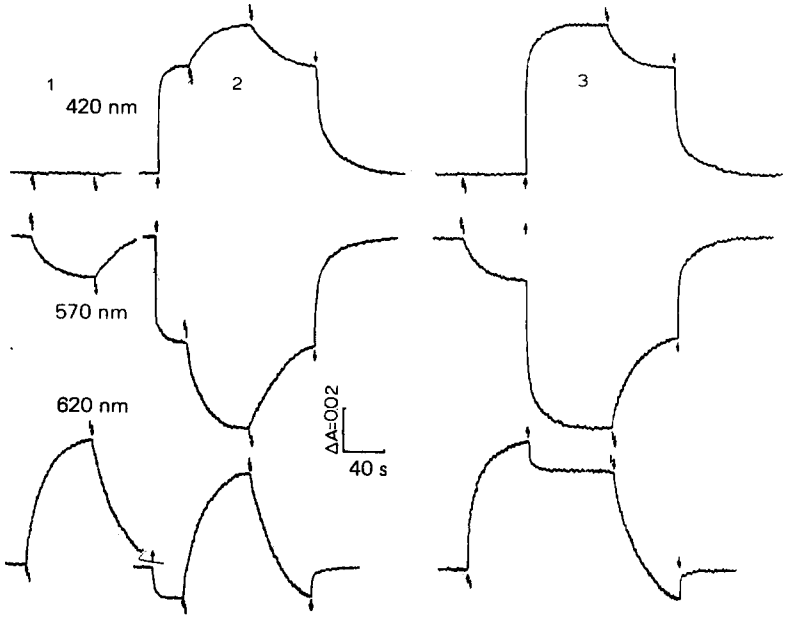

Fig. 1. The kinetics of electric- and photo-induced absorbance changes in purple membrane films from *H. halobium*. An aqueous suspension of the preparation (5—7 mg BR/ml, pH 7.2) was dried on a glass slide covered with a translucent aluminum film; drying at room temperature and at 0.1 torr pressure. The other electrode was provided by evaporating a thin aluminum layer in vacuum upon the surface of the dried preparation. Sample thickness as calculated from its electric capacity was $5 \cdot 10^{-6}$ m. The absorbance of a film at 568 nm was 1.2 units. ↿↑, Arrows up, electric field or light irradiation respectively switched on; ↿↓, arrows down, switched off. The electric field strength, E, was $2 \cdot 10^7$ V $\cdot$ m^{-1}. The irradiance of blue-green illumination (380—580 nm) was 80 W $\cdot$ m^{-2}. Measurements were taken at +17°C and at a relative humidity of 60%.

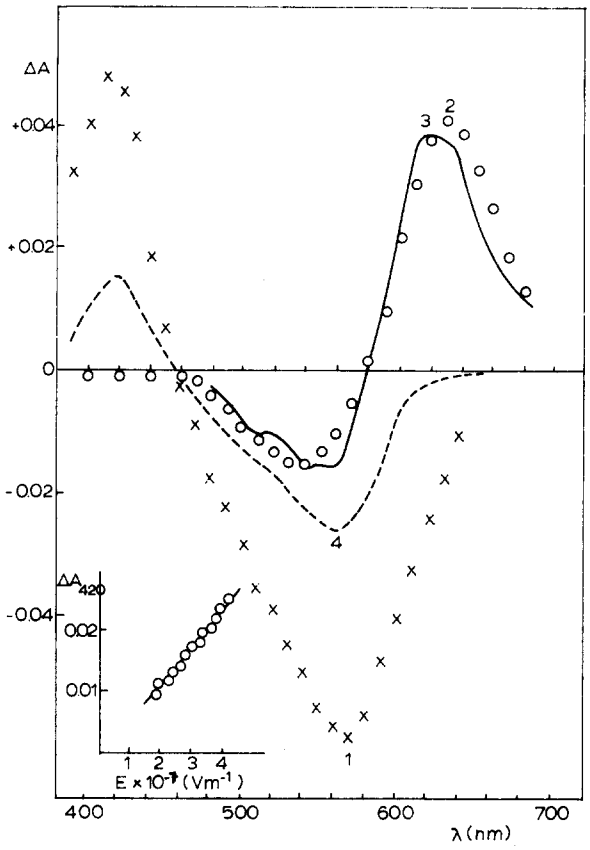

Fig. 2. The spectra of photo- and electric-induced absorption changes in purple membrane films from *H. halobium*. X, 1, light-induced changes (380—580 nm, 80 W · m^{-2}); ○, 2, changes induced by an external electric field ($2 \cdot 10^7$ V · m^{-1}) applied in the dark; ———, 3, calculated electrically-induced changes; - - - - - -, 4, the difference of the electrically-induced absorption changes in the light and dark. The insert shows the field dependence of an enhancement of the light-induced absorption change at 420 nm. Other conditions as in Fig. 1.

which induced the $BR_{570} \rightarrow BR_{412}$ transition, stimulated the photo-induced absorbance changes in this spectral region (version 2). It can be seen from the comparison of the two experiments (versions 2, 3) that the effect is the same irrespective of whether electric field or light was applied first. The differential spectrum of Fig. 2. demonstrates results of the electrical stimulation of photo-induced absorbance changes (curve 4). This spectrum (the difference in amplitude of the electrically-induced absorbance changes in the light and dark) is close to that for the $BR_{570} \rightarrow BR_{412}$ transition in the normal BR_{570} photocycle (curve 1). The insert in Fig. 2 shows the field dependence of an enhancement of ΔA_{420} to be linear. The electrically-induced changes of the BR absorbance in the dark (ΔA_{630}) and in the light (ΔA_{420}) follow virtually the same kinetic pattern (Fig. 1), with a half-time of rise and decay of about 10—20 s. Their time-courses are functions of field strength, temperature and relative humidity of the preparation [1—3].

The amplitudes and kinetics of ΔA_{630} and ΔA_{420} are independent of field

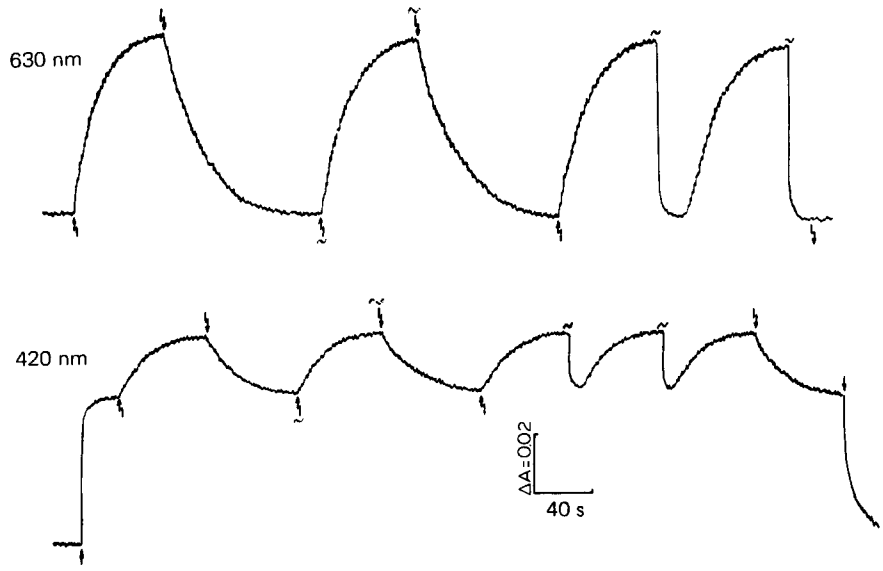

Fig. 3. The kinetics of electrically-induced absorption changes in purple membrane films from *H. halobium* in the dark (ΔA_{630}) and in the light (ΔA_{420}) upon reversion of field polarity. ↑↓, field on and off, respectively; ↑̲↓̲, reverse field on and off; ~, reversion of field polarity. Other conditions as in Fig. 1.

polarity (Fig. 3), but if the field polarity is converted when the steady-state level of ΔA_{630} or ΔA_{420} is reached, the amplitude decreases rapidly ($t_{1/2} < 0.5$ s) to zero, then gradually rises again to the steady-state level, following its characteristic kinetic pattern (Fig. 3).

As suggested in Refs. 15 and 16, photoexcitation causing a marked change in the dipole moment of retinal [17] initiates polarization changes in its vicinity. The latter leads to fast reversible conformational rearrangements in the pigment-protein matrix of BR, involving a proton release on one side of the membrane — during BR_{412} formation — and proton uptake on the opposite side, during its decay [18,19].

Our findings indicate that the formation of the BR_{412} intermediate in the BR_{570} photocycle ($BR_{570} \rightarrow K \rightarrow BR_{412}$) can be accompanied by changes in position of charged, polar and polarizable groups in BR. The reversion of field polarity leads to the fast relaxation of the preformed polarized state and to the disappearance (with $t_{1/2} < 0.5$ s) of the electrically induced absorbance changes (Fig. 3); the amplitudes of ΔA_{630} and ΔA_{420} decline nearly to zero. The simultaneous polarization in the opposite direction (in accordance with the new direction of the field) occurs for much longer times ($t_{1/2} = 10$—20 s) and generates a similar spectral state in the other, oppositely oriented fraction of BR. Equal amplitudes of the steady-state absorbance changes in both cases (Fig. 3) indicate that these oppositely oriented bulks of purple membranes are equal, as was supposed in Materials and Methods.

So, the external electric field of an appropriate direction, interacting with field-susceptible groups (charged, polar, polarizable) may affect also those involved, in some way or other, in the translocation of protons across the purple membrane. The most likely candidates for this role are two residues of

the aspartic acid adjacent to the lysine-41 residue, to which the retinal chromophore is linked in the BR macromolecule, several tryptophan residues being apparently close to retinal [20,21].

Our data clearly illustrate the importance of polarization processes in the BR functioning. Further experiments may evidently help to elaborate definite schemes of the processes in terms of molecular dynamics in the retinal vicinity.

Electret-thermal analysis of purple membrane dry films

Previously it was demonstrated that purple membranes can be polarized by an external electric field and that this polarization can be retained for a long time [1,2]. In this paper we present more thorough study of the phenomenon.

Purple membrane films with built-in electrodes, similar to those described above, were subjected to a constant electric field of $2 \cdot 10^7$ V · m^{-1} at room temperature. One should expect changes in the equilibrium positions of various electrically susceptible groups as a result of interaction with the field. In the presence of the field the sample was cooled down to —160° C during 8—10 min to fix it in the polarized state. The field was then removed and the sample was gradually heated at a rate of 7 K/min. The thermodepolarization current flowing in the external electric circuit was measured as a function of temperature.

Fig. 4 represents the typical thermodepolarization current curve for a dry film of purple membranes. The current passes through three maxima at —130, —100 and +30° C. Moreover, a less distinct maximum at —50° C can be seen.

The maxima reflect, evidently, the relaxation of different groups interacting with the field. Obviously, low-temperature maxima reflect the reorganization of the most mobile groups. In fact, the character of the low-temperature part of the curve was unaffected by decreasing of the temperature of polarization from +20° C to —130° C. In this case only the integral of the current was found to be somewhat smaller, without a change in the position of the maxima,

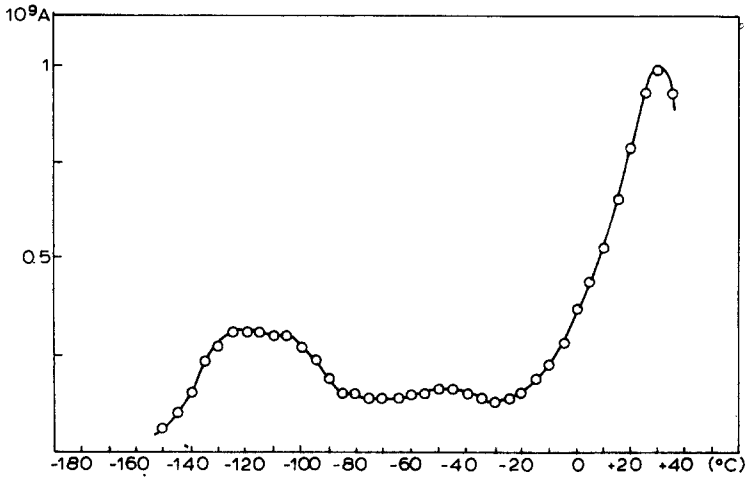

Fig. 4. The curves of thermodepolarization currents in purple membrane films from *H. halobium*. Samples were as in Fig. 1. A sample exposed to an electric field ($2 \cdot 10^7$ V · m^{-1}) was cooled in the dark to —160° C; the field was then removed and the sample was heated at a rate of 7 K/min.

indicating dipole reorientation [22]. Thus, it is possible to suggest that the low-temperature current peaks reflect the relaxation of mobile groups, for instance, those in the vicinity of retinal, and, probably, of retinal itself.

Photo-induced batho-BR state, K, is known to be stable at cryogenic temperatures [4,5] (see also the review, Ref. 19) and could be associated with local polarization around excited retinal chromophore. On warming up to —140—130°C, K spontaneously converts to the subsequent other forms of BR. It is interesting that the low-temperature peaks of thermodepolarization current were observed at just those very temperatures.

The fast (microsecond) reorientation of retinal in the pigment-protein complex of BR after the exposure to an electric field pulse was demonstrated in Ref. 23.

On the other hand, the maximum of current at +30°C disappeared when the temperature of polarization was lowered to only 0°C. Thus, this peak may be related to the electrically-induced reorganization of less mobile structural components of the purple membrane, for instance, of some protein segments of the BR macromolecule (see Ref. 24 and our work on photosynthetic membranes and their fragments [13,14]).

To conclude, it is worth mentioning that the fields used were of the same order of magnitude as those normally encountered in the bioenergetic membranes [25]. Since BR is an integral membrane protein [19], it is obviously subjected to such fields in situ. Whether it is important to its functioning is not clear yet.

Acknowledgements

Included in the program of scientific research 'Rhodopsin', with Academician Yu.A. Ovtchinnikov in charge. We are grateful to V.P. Skulachev, corresponding member of the U.S.S.R. Academy of Sciences, for valuable remarks and discussion of the results. We thank Dr. L.N. Tchekulaeva for fragments of purple membranes *H. halobium* R_1.

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