SURFACE CHARGE MOVEMENTS OF PURPLE MEMBRANE DURING LIGHT-DARK ADAPTATION

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ABSTRACT The difference in the surface charge distribution between light-adapted and dark-adapted purple membranes was investigated with electric dichroism measurements from approximately pH 5 to pH 11. Purple membrane sheets in solution are oriented in a weak electric field by their permanent dipole moment, which is due to the charge distribution of the membrane surfaces and/or within the membrane. The degree of orientation of purple membrane sheets was obtained from the measurement of "electrical anisotropy" of retinal chromophore in the membranes. At about pH 7, there was no difference in the "electric anisotropy" between light- and dark-adapted purple membranes. At about pH 9, the electric anisotropy of dark-adapted purple membrane was larger than that of light-adapted purple membrane. But at around pH 6 the difference was opposite. Linear dichroism experiments did not show any change of retinal tilt angle with respect to the membrane normal between the two forms from approximately pH 5 to pH 10. This result indicates that the changes in the "electric anisotropy" are not due to the change of retinal tilt angle, but due to the change in the permanent dipole moment of the membrane. To estimate the change in surface charges from the permanent dipole moment, we investigated the difference of the permanent dipole moment between the native purple membrane and papain-treated purple membrane in which negative charges in the cytoplasmic-terminal part are removed. This estimation suggests that this light-dark difference at around pH 9 can be accounted for by a change of ~0.5 electric charge per bacteriorhodopsin (bR) molecule at either of the two surfaces of the membrane. We also found from pH electrode measurements that at about pH 8 or 9 light adaptation was accompanied by an uptake of ~0.1 protons per bR. A possible movement of protons during light-dark adaptation is discussed. The direction of the permanent dipole moment does not change with papain treatment. The permanent dipole moment in papain-treated purple membrane is estimated to be 27 ±2 debye/bR.

INTRODUCTION

Purple membrane exists in the plasma membrane of *Halobacterium halobium* and functions as a light-driven proton pump (1, 2). The membrane contains the protein bacteriorhodopsin (bR) with retinal as its chromophore (3). In the dark, bR exists in a "dark-adapted" form (bR^D) with the absorption maximum near 560 nm. After exposure to yellow light, it becomes "light-adapted" (bR^L) with the absorption maximum near 570 nm (4).

Investigation of electrical anisotropy properties of bR may be helpful in understanding the mechanism of proton pumping. From electric dichroism measurement the permanent dipole moment, which is due to the charge distribution of the membrane surfaces and/or within the membrane, can be investigated (5–9). The permanent dipole moment changes with the surface charge changes that are affected by changes in *pK*, pH, and ion concentration (9).

Light-dark adaptation events might be related to the proton pumping events. Isomerization of retinal is an essential event in the proton pumping system. It is known that light-dark adaptation also involves the isomerization of retinal. It has been commonly thought that bR^L has only all-*trans* retinal and that bR^D has retinal in equal proportions of the all-*trans* and 13-*cis* forms.

Equilibrium and kinetic aspects of light-dark adaptation are sensitive to pH (10). Recently, Ohno et al. reported that the ratio of all-*trans* to 13-*cis* retinal in bR^D is also a function of pH (11). It may be that light-dark adaptation involves *pK* changes of some amino acid residues. In addition, at low humidity light adaptation does not proceed to completion (12). Light-dark adaptation thus also seems very sensitive to hydrogen ion mobility.

MATERIALS AND METHODS

Purple membrane was purified from *Halobacterium halobium* strain Et1001 according to the established procedure (13). The sample was then washed in 10 mM EDTA to decrease the ion screening effect, and finally washed four times in water with resistivity >17 megohm-cm.

The electric dichroism apparatus and measuring procedures were described in the previous report by Kimura et al. (9), except for the following modifications. The pH was controlled by various 5 mM buffer

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solutions (pH 5–7 Mes, pH 6.5–9 Hepes, pH 8.5–10.5 Tris), and the temperature was 10°C for all measurements unless otherwise stated.

We left the sample in the dark overnight at room temperature before measuring the electric and linear dichroism of the dark-adapted form. After exposure to yellow light, we measured the electric dichroism of the light-adapted form. The completion of dark and light adaptation was ascertained from absorption spectrum. Lastly, we measured the pH (±0.1) with a pH meter (Horiba Corp., Kyoto, Japan).

The difference in the retinal tilt angle between bR^L and bR^D was investigated by a variation of the method of Heyn et al. (14). Oriented purple membrane films were prepared by electrophoresis according to Varo (15). Linear dichroism measurements were then done in the same buffer solution as in the electric dichroism measurements. When soaked in an acidic buffer, the color of the film turned blue showing that the interior of the film also became acidic. This change in color was very fast as in a purple membrane suspension. The purple membrane film in the buffer solution did not dissolve at least for 5 h; the orientation of the membrane was preserved as evidenced by x-ray diffraction measurements (T. Furuno, personal communication). Absorbance was measured at λ = 530 nm, an isosbestic wavelength of bR^L and bR^D. Other experimental conditions were the same as for the dielectric dichroism measurements.

The pH change of purple membrane suspensions with light adaptation was measured under Ar or N_2 gas flow using light insensitive pH electrodes. After the pH change measurement, we calculate the number of protons taken up, per bR, from titration measurements with NaOH or HCl at each pH. The apparatus and measuring procedures are described by Ohno et al. (16) for details.

Papain treatment was done according to Renthal et al. (17). A solution of 0.05 mg/ml papain in deionized water (0.12 ml) was mixed with 0.2 ml of activation solution (0.05 M cysteine, 0.02 M EDTA, pH 8.0) and added to 1.0 mM of purple membrane suspension (containing ~60 nmol of bR) in 0.05 M Tris, pH 8.0. The reaction mixture was incubated at 40°C. After the reaction, the sample was diluted with ~50 volumes of cold water and washed with water four times. After each papain treatment the sample was monitored by SDS polyacrylamide gel electrophoresis.

PRINCIPLES OF ELECTRIC DICHROISM

Purple membrane sheets in solution are oriented by an electric field. Under a constant or low-frequency field the torque originates mainly from the interaction of the membrane's permanent dipole moment, which is parallel to the membrane normal, with the applied field. The membranes are oriented perpendicularly to the field. On the other hand, at high frequency the torque due to the permanent dipole moment is effectively averaged out. Then the membranes have a parallel orientation to the field. The torque at high frequency comes from the induced dipole moment, which is due to the polarization of ions in the atmosphere near around the membrane.

The degree of orientation under an electric field is assessed by measuring the anisotropy of the absorption by retinal in the purple membrane. The "electric anisotropy", $S_\parallel - S_\perp$, is given by

$$S_\parallel - S_\perp = \frac{A_\parallel(E) - A_\perp(E)}{A}, \tag{1}$$

where A is the absorption in the absence of electric field and $A_\parallel(E)$ and $A_\perp(E)$ are absorptions in the presence of electric field. The subscripts $\parallel$ and $\perp$ mean that the absorption was measured by using polarized light, parallel and perpendicular to the direction of the electric field.

The electric anisotropy of purple membrane suspensions has been shown to depend on two factors as

$$S_\parallel - S_\perp = \Theta \cdot \Psi. \tag{2}$$

The angle factor Θ is given by the tilt angle γ of the absorption transition moment of retinal with respect to the membrane normal as

$$\Theta = 3\,\frac{3\cos^2\gamma - 1}{2}. \tag{3}$$

The retinal is attached tightly to the bR and is immobile, if the electric field applied to the membrane is very low (<100 V/cm). The tilt angle γ is reported to be ~70° (14). We also found that the retinal tilt angle with respect to the membrane normal of light- and dark-adapted purple membrane is almost the same (see Results, below). Thus the factor Θ in Eq. 2 can be regarded as a constant (−0.9736 for γ = 70°). Any change in the electric anisotropy reflects a change in the orientation factor Ψ.

The orientation factor, Ψ, is the ensemble average of a function of the angle θ between membrane normal and the direction of the applied field as follows:

$$\Psi = \left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle. \tag{4}$$

In the case of disklike particles, of which permanent dipole moment μ is perpendicular to the disk surface and polarizability α is along the disk surface, the orientation factor ϕ is approximately given by

$$\phi = \frac{1}{15}E^2\left(\frac{\mu^3 - \alpha}{kT}\right) \tag{5}$$

when the intensity E of the applied electric field is sufficiently low. Then the slope in a $(S_\parallel - S_\perp) \sim E^2$ plot allows the estimation of the magnitude of permanent dipole moment or polarizability of the membrane. The permanent dipole moment was estimated at low frequency electric field where the orientation is induced by pure permanent moment, whereas the polarizability α was estimated at high frequency field, where the membrane is oriented solely by induced dipole moment (9).

The permanent dipole moment depends on the size of the membrane, and is basically proportional to the area of the membrane sheet. One can calculate the permanent dipole moment per bR by the following procedure. After the applied field is switched off, the electric anisotropy decays with the rotational relaxation time τ of the membrane sheets. According to Perrin (18), the rotational relaxation time of τ of a disk is expressed by

$$\tau = \frac{1}{6D} = \frac{16\eta c^3}{9kT}, \tag{6}$$

where c is the radius of the disk, η is the medium viscosity, k is the Boltzmann constant and T is the temperature. If

one assumes that the purple membrane sheet is approximated by a rigid disk, one can estimate its diameter and thus the number of bR molecules in the sheets.

RESULTS

"Electric Anisotropy" Change Between bR^L and bR^D at Different pHs

Fig. 1 shows the difference between the electric anisotropy of bR^L and bR^D at different pH values. The electric anisotropy of bR^L is larger than that of bR^D at pH 6.1, but is smaller than bR^D at pH 8.5. The difference disappears at pH 7.0.

Fig. 2 shows the change of the electric anisotropy during the light and dark adaptation at pH 7.2 and at pH 8.7. The kinetics of the light/dark adaptation was followed by the absorption change at 570 nm at 30C; at both pH values the half time of dark-adaptation was ~45 min, as reported by Ohno et al. (10). Fig. 2 *b* shows that the change in the electric anisotropy at pH 8.7 is clearly dependent on the light-dark adaptation, and is reversible.

Retinal Angle of bR^L and bR^D in the pH Range from 5 to 10

As discussed in Principles, the electric anisotropy of a purple membrane suspension depends on two factors: the orientation factor of purple membrane and the retinal tilt angle against the axis normal to the membrane surface. The observed changes between bR^L and bR^D, therefore, could have resulted from a change in the retinal tilt angle.

Changes in the retinal tilt angle between bR^L and bR^D were estimated by the linear dichroism measurements described in Materials and Methods. However, we found

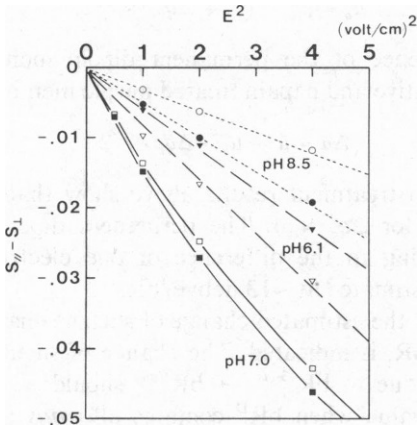

FIGURE 1 The difference of electric anisotropy ($S_\parallel$–$S_\perp$), plotted against the square of the applied electric field (E^2), between light-adapted (▽ □ ○) and dark-adapted (▼ ■ ●) purple membranes (OD_{570} ~ 0.3) at various pH values; pH 6.1 ▽ ▼, — — —), pH 7.0 □ ■, —) and pH 8.5 (○ ● ---). All measurements were performed with reversing rectangular electric fields at 10°C.

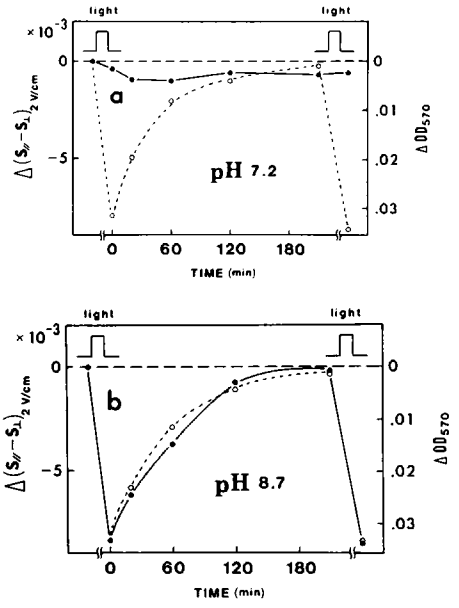

FIGURE 2 The change of electric anisotropy ($S_\parallel$–$S_\perp$), (●, —) at 2 V/cm electric field of reversing rectangular pulse during light and dark adaptation at 30°C and at pH 7.2 (*a*) and 8.7 (*b*). Light-dark adaptation was followed by the absorption change at wave length 570 nm (○, ---). Light was applied enough for light adaptation.

that there were no differences in the tilt angle between bR^L and bR^D in the pH range from 5 to 9 (Fig. 3). We also measured the linear dichroism continuously during light-dark adaptation, but did not detect any change even at pH 8.7 where the electric dichroism showed a large time dependence (Fig. 2). If the change in the electric anisotropy at pH 8.7 had been due to a change in the retinal tilt angle, the tilt angle should have changed by more than 5°. Fig. 3 clearly shows that the difference in the electric anisotropy must be attributed to a difference in the electric permanent dipole moment.

In the pH range from 5 to 10, the absolute value of the retinal tilt angle in bR^L and bR^D did not change significantly (data not shown). Thus the retinal tilt angle is

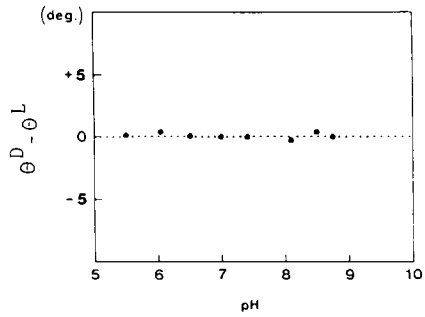

FIGURE 3 The difference of retinal tilt angle with respect to membrane normal between bR^L and bR^D in the pH range from 5 to 9 at 10°C obtained by linear dichroism measurements (see Materials and Methods).

almost the same at any pH, and both in the light- and dark-adapted bR.

Permanent Dipole Moment Difference Between bR^L and bR^D, as a Function of pH

The electric anisotropy is proportional to the square of the electric field strength when the electric field is weak (Fig. 1). So we calculated the permanent dipole moment, per bR, from the initial slope in the $(S_\parallel - S_\perp) \sim E^2$ plot, and the rotational relaxation time of the membrane sheets (see Principles). Fig. 4 shows the difference of the permanent dipole moment between bR^L and bR^D in the pH range 5 to 11.

Permanent Dipole Moment Change Corresponding to the Difference of one Surface Electric Charge: Estimation by Papain Treatment

Renthal et al. (17) reported that 22 amino acids from the COOH-terminus of bR molecules were cut off by papain treatment and four negative charges were removed from the cytoplasmic side of the purple membrane. So we estimated the change in the permanent dipole moment by the papain treatment.

When the purple membrane was treated with papain, the permanent dipole moment of bR^L, measured at pH 7.0, decreased. Fig. 5 shows the change of the permanent dipole moment, per bR, as a function of time after the papain was introduced. After 360 min of papain treatment, the value of the permanent dipole moment, per bR, was constant at 27 ± 2 debye, and we confirmed from SDS gel electrophoresis measurements that the molecular weight of bR decreased by ~2,000 D (data not shown). Under these conditions, four negative charges had been cut off from the cytoplasmic side (17, 19).

Hence the permanent dipole moment decrease monotonically without becoming zero during the removal of four negative charges from the cytoplasmic side, the direction of the permanent dipole moments of both native and papain

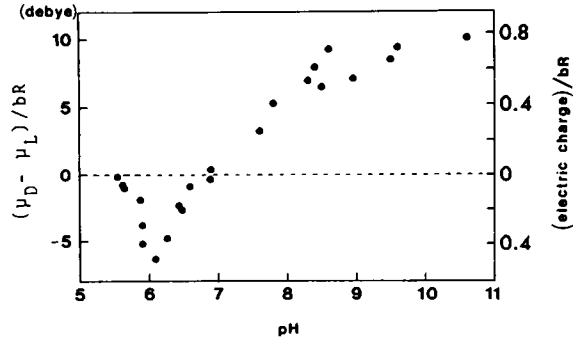

FIGURE 4 pH dependence of the difference in permanent dipole moment (*left vertical axis*), per bR, between bR^L and bR^D at 10°C. Right vertical axis indicates the change of surface charge difference, per bR, estimated from papain treated purple membrane. See text for details.

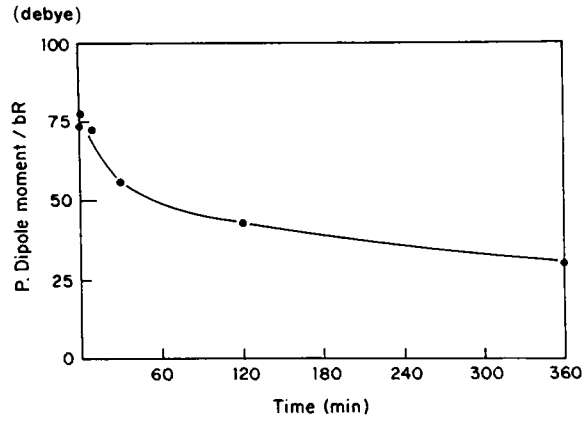

FIGURE 5 The change of the permanent dipole moment, per bR, as a function of time after the incubation with papain at 10°C and pH 7.0. Change in mol wt was checked by SDS gel electrophoresis measurement.

treated purple membranes should be from the cytoplasmic side to the outer side.

If we assume that every charged group of purple membrane is located in either of the two surfaces, the net charge q and the permanent dipole moment (μ) of the membrane per bR are given by

$$q = q_e + q_c \tag{7}$$

$$\mu = (q_e - q_c) \cdot \frac{r}{2}, \tag{8}$$

where q_e and q_c are the total charges per bR of the extracellular and cytoplasmic surfaces. For the thickness of the purple membrane r, we put 40 Å in the following calculation. Papain treatment changes only the cytoplasmic surface charges. The value of the permanent dipole moment of papain treated purple membrane (μ_p) is given by

$$\mu_p = \{q_e - (q_c + \Delta q_c)\} \cdot r/2. \tag{9}$$

The difference of the permanent dipole moment ($\Delta\mu$) between native and papain treated purple membranes is

$$\Delta\mu = \mu - \mu_p = \Delta q_c \cdot r/2. \tag{10}$$

The papain treatment results above show that $\Delta\mu = 52$ debye/bR for $\Delta q_c = 4$. The permanent dipole moment corresponding to the difference of one electric surface charge is estimated at ~13 debye/bR.

In Fig. 4 the estimated change of surface charge difference, per bR, is indicated. The change of surface charge difference due to $bR^{13\text{-}cis} \rightarrow bR^{trans}$ should be twice the indicated value when bR^D contains all-*trans* and 13-*cis* retinal in equal proportion.

Proton Uptake with Light Adaptation Between pH 5 and 10

Fig. 6 shows the light-induced pH changes in purple membrane solutions at pH 8.3 and 6.9. By the irradiation

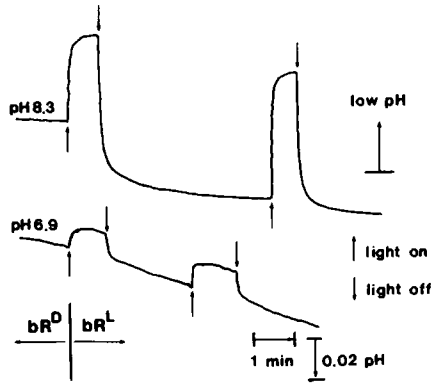

FIGURE 6 Light-induced pH changes of purple membrane solutions in 0.2 M KCl at pH 8.3 and 6.9 (15°C). See text for details.

with yellow light, dark-adapted purple membrane, bR^D, changes into a photo-steady-state containing mostly bR^L with some intermediate states. The decrease in pH under irradiation indicates the increase of M intermediate state. After the irradiation, bR is in light-adapted form. When the initial pH of a bR^D solution is 8.3, the pH of the bR^L solution after the irradiation become more alkaline than that of the initial solution; that is, protons in solution are taken up by the membrane with light adaptation. When the initial pH of a bR^D solution is 6.9, there is no pH difference between the bR^L solution after the irradiation and the initial bR^D solution except the monotonous drift of pH.

Fig. 7 shows the pH dependence of proton uptake by the membrane with light adaptation in 0.2 M KCl, and without salt. In the pH range from 7 to 10, proton uptake with light adaptation is not affected by salt.

Polarizability Ratio Between bR^L and bR^D as a Function of pH

Electric anisotropy in a sinusoidal electric field was measured at a higher frequency, 150 Hz. For a weak electric field, it depends on the ionic polarizability of purple membrane, that is, the displacement of surrounding ions along membrane surface (9).

Fig. 8 shows the ratio of the electric anisotropy of bR^L and that of bR^D in the pH range from 5 to 10. Above pH 6.5 the electric anisotropy, and therefore the polarizability

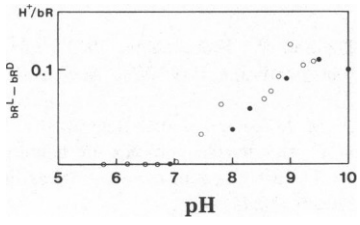

FIGURE 7 pH dependence of proton uptake with light adaptation; •, 0.2 M KCL at 15°C; ○, without salt at 40°C. The number of protons was calculated from the pH titration measurements at each pH.

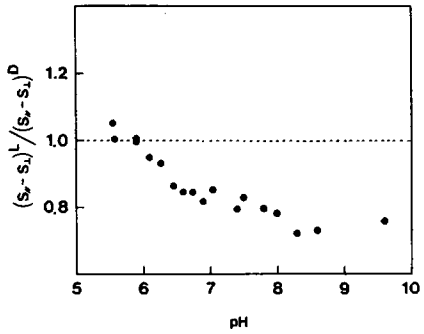

FIGURE 8 pH dependence of the ratio (bR^L/bR^D) of electric anisotropy due to induced dipole moment. Electric anisotropy was measured in the sinusoidal electric field of 150 Hz, at 10°C.

of the bR^L is smaller than that of bR^D. The ratio of bR^L to bR^D is 0.8 ±0.05 in the pH range from 6.5 to 10.

DISCUSSION

It is commonly known that one difference between bR^L and bR^D is the isomerization state of retinal. bR^L contains only all-*trans* retinal, and bR^D contains all-*trans* and 13-*cis* retinal in equal proportion. Recently Bagley et al. (20) and Rothschild et al. (21) reported that there may be conformational changes of protein between bR^L and bR^D, detected by Fourier transform infrared difference spectroscopy (FTIR) measurements of absorption lines related to carboxyl group. Moreover, Tsuda et al. (22) reported that the molar volume change between $bR^{all\text{-}trans}$ and $bR^{13\text{-}cis}$ was −7.8 ±3.2 ml/mol from high-pressure absorption spectra, and suggested a difference of dissociation state of one or more amino acid residues of bR between bR^L and bR^D. Other spectroscopic and enthalpy studies (4, 23–25) did not report any conformational changes between bR^L and bR^D. The only precise difference between bR^L and bR^D that is known is the isomerization state of retinal.

The difference of the permanent dipole moments between bR^L and bR^D at various pH probably reflect a change of surface charge distribution. It is difficult to imagine that the rather large difference between the two forms of bR is due only to charge distribution changes that occur due to a large conformational change of the protein. If there were a large conformational change and surface charge redistribution, the tilt angle of retinal would be expected to change substantially.

Our observed change of charge distribution is probably due to the change of dissociational states of surface amino acids of bR between bR^L and bR^D. Specifically we suppose that the isomerization of retinal might shift the *pK* value of some amino acid residues of bR. Because the difference of the permanent dipole moment between bR^L and bR^D is rather large and very sensitive to pH, we think that the amino acids involved are close to the surfaces of the membrane. There are some ionizable amino acid residues inside the membrane, but these can make rather small changes in the permanent dipole moment. Therefore we

suppose that the pK values of some ionizable amino acid residues at the membrane surfaces change during the light-dark adaptation. We also detected the net uptake or release of membrane protons with light adaptation (Fig. 7). This result supports the suggestion that the change of pK values occurs during dark/light adaptation.

The change of surface charge during light adaptation, estimated from the electric dichroism measurements and calibrated via papain treatment, is ~0.5–0.6 electric charge per bR at about pH 8 or 9 (Fig. 4), but the net uptake of protons as estimated from the pH electrode measurements is 0.1/bR at same pH range (Fig. 7). From pH 7 to 9, the trend of pH dependence of the surface charge difference qualitatively agrees with the net uptake of protons. However, the pH dependences are quite different at pH 5 to 7.

If the difference between the two adapted forms is due only to the change of the number of protons at the both surfaces of membranes, we can calculate this from the results of electric dichroism and pH electrode measurements. The change of permanent dipole moment per bR, $\Delta\mu$/bR, and total decrease of protons in solution per bR, ΔN_H/bR, are described by

$$\Delta\mu/\text{bR} = (\mu_L - \mu_D)/\text{bR} = (\Delta q_e - \Delta q_c) \cdot r/2$$

$$\Delta N_H/\text{bR} = \Delta q_e + \Delta q_c,$$

where Δq_e and Δq_c are numbers of protons taken up at each surface per bR. Then the values of Δq_e and Δq_c are estimated to be -0.2 and $+0.3$ at about pH 8–9, and are $+0.2$ and -0.2 at about pH 6, respectively. Namely, during light adaptation, protons were taken up into cytoplasmic side and were released from extracellular side at about pH 8–9, but the exactly opposite at around pH 6.

From the polarizability change due to light-dark adaptation, we can estimate the probable change in the total number of charged sites on both surfaces. When the number of charged sites on both surfaces decreases, the polarizability also decreases. From Fig. 8, the polarizability of bR^L is smaller than that of bR^D in the pH range from 6.5 to 10. In this pH range, the total surface charge of bR is negative. Therefore the smaller polarizability of bR should be due to the decrease of surface negative charge compared with bR^D. In this manner, we can also confirm the proton uptake during light adaptation observed by pH measurements.

Thus, we guess that the pK of several amino acids of the bR is affected by the isomerization of retinal that occurs in light-dark adaptation. This presumption may be suggestive of the study of proton pumping that also occurs with the isomerization of retinal.

Above we have assumed that the change of the permanent dipole moment in light-dark adaptation is due only to a change of protonation on one or both surfaces. The electric dichroism measurement, however, is sensitive to more than the release and/or uptake of protons. We cannot exclude the possibility that other ions are released from and/or taken up on the surfaces of bR during light-dark adaptation.

The retinal angle with respect to the membrane normal did not change ($< \pm 0.5°$) between bR^L and bR^D in the pH range 6–10. We could not detect the very small change of tilt angle due to the light adaptation (0.4°) reported from electric dichroism measurement (26). In our measurement, we only measured the difference in the tilt angle of retinal between bR^L and bR^D on the same oriented film of purple membrane in the pH range from 5 to 10. Therefore relative accuracy of the result should be much higher than that of ordinary electric dichroism measurements.

Kimura et al. (9) measured the electric dichroism of trypsin treated purple membrane, in which one negative charge was removed from the cytoplasmic side. They concluded that the negative charge corresponded to ~24 debye/bR. From our papain treatment of purple membrane, we calculate that one negative charge corresponded to ~13 debye/bR.

The origin of the discrepancy may be because the value of the permanent dipole moment is proportional to the difference between charges of the two surfaces, and to the distance between them. The permanent dipole moment change due to trypsin treatment involves the charge farthest from the membrane surface. In contrast, the permanent dipole moment change due to papain treatment represents the average effect of the four negative charges of the cytoplasmic side. Therefore the permanent dipole moment change per charge estimated from trypsin treatment is larger than that estimated from papain treatment. Our calibration should be better for the estimation of surface charge difference.

We thank Dr. Y. Kimura for helping our experiments and very helpful suggestions. We also thank Dr. K. Kinosita, Dr. T. Kouyama, Dr. M. Fujiwara, and Dr. J. Marque for their helpful discussions. We thank Dr. T. Furuno for his x-ray measurements.

This work was supported by special coordination funds for promotion of science and technology and a research grant for Solar Energy-Photosynthesis given by the Agency of Science and Technology of Japan.

Received for publication 16 September 1985 and in final form 20 February 1986.

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