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Formation of blue membrane of bacteriorhodopsin by addition of tetrakis(4-fluorophenyl)boron, an hydrophobic anion

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Bacteriorhodopsin is known to lose its purple color and turn blue when it is either acidified below pH 3.2 or deionized. We have found that a lipophilic anion, tetrakis(4-fluorophenyl)boron ($TFPB^-$) changed the color of bacteriorhodopsin from purple to blue even at neutral pH. $TFPB^-$ can mimic the action of H^+, although its charge is opposite. Addition of low concentrations of lipophilic cation into deionized blue membranes restored the color to purple. These results were analyzed in terms of the change in surface potential caused by the binding of lipophilic anions and cations. Analysis with Gouy-Chapman theory suggested the presence of a specific binding site which is hydrophobic and located near the retinal. It seems that the surface potential in the local surface domain covering this binding site is different from that of a whole membrane surface, and affects the binding of lipophilic ions to the site. The change in the electrical potential nearby the binding site caused by the binding of the lipophilic ion is considered to change the dissociation of color-controlling group(s) and to induce the color conversion of the membrane.

Introduction

Bacteriorhodopsin is the only protein in the purple membrane of *Halobacterium halobium* [1,2]. Bacteriorhodopsin is a retinal protein. Although retinal itself has an absorption maximum (λ_{max}) at 370 nm, λ_{max} of bacteriorhodopsin is 560–570 nm, depending on the dark- or light-adapted form. Retinal forms the protonated Schiff base with the lysine of the protein. This positive charge and other negative charges of dissociable amino acid residues in the vicinity of the chromophore are considered to cause a large red shift of the main absorption band [3].

Oesterhelt and Stoeckenius [4], and Moore et al. [5,6] reported that the purple membrane changes its color to blue at low pH (below pH 3.2) due to a red-shift of the absorption band to 605 nm. The red-shifted species are called blue membrane. Kimura et al. [7] de-ionized the purple membrane by passing it through a column of cation-exchange resin and obtained the blue membrane. Ebrey and his colleagues [8,9] showed that extensively washed purple membrane has about 1 Ca^{2+} and 3–4 Mg^{2+} bound per bacteriorhodopsin molecule. When these divalent cations are re-

Abbreviations $TFPB^-$, tetrakis(4-fluorophenyl)boron, TPP^+, tetraphenylphosphonium, TPB^-, tetraphenylboron, Mops, 4-morpholinepropanesulfonic acid, Mes, 4-morpholineethanesulfonic acid

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moved by a variety of washings, bacteriorhodopsin turned to blue. By addition of various inorganic cations, the original purple color was restored [7–9].

In the present paper, we show that tetrakis(4-fluorophenyl)boron ($TFPB^-$), a lipophilic anion, changes the color of bacteriorhodopsin from purple to blue even at neutral pH and that addition of a lipophilic cation into the blue membrane restores the purple color. Results obtained are interpreted in terms of the local surface potential change which leads to the change in the proton concentration in a local domain nearby color-controlling dissociable groups located in the vicinity of the chromophore. The significance of the surface potential in the local domain, as shown in the functioning of a variety of membranes [10–13], is pointed out.

Materials and Methods

Purple membrane was prepared from *Halobacterium halobium* strain S-9 cells according to the standard method [14]. The purified purple membrane was washed several times with distilled water (pH ≈ 6) and the sample remained purple. Deionized blue membrane [7] was obtained by passing through a column of cation exchange resin (MWC-1, Muromachi K.K., Tokyo). The spectrum of the blue membrane obtained was shown in Fig 4. The pH of the blue membrane after passing the column was 4.5–5.5, depending on the bacteriorhodopsin sample used. $TFPB^-$ (sodium salt), TPB^- (sodium salt) and TPP^+ (chloride salt) were purchased from Dojin Chem. Lab. (Kumamoto) and used as delivered. Other chemicals used were analytical grade. The buffer used were Mops for pH 7.0, Mes for pH 6.0 and citrate for pH 4.5.

Absorption spectra were obtained with a UV-300 photospectrometer (Shimazu, Kyoto) which is designed for the measurement of turbid samples. The temperature of the sample was maintained at 25 °C by passing thermostated water through the cuvette holder. The spectroscopical pH titration of bacteriorhodopsin was performed with a laboratory-made cuvette with which a stirring equipment and a glass electrode (No. 6028, Hitachi-Horiba) were installed. The glass electrode was connected to a pH meter (F7AD, Hitachi-Horiba). In acidic solution below pH 2.5–2.0, bacteriorhodopsin aggregated but the spectra were obtained successfully under vigorous stirring. Flash photolysis was performed with an apparatus with Xe-flash (duration approx. 20 μs) as described previously [15]. The excitation flash was provided through a combination of a cut-off filter (> 520 nm) and an interference filter of 570 nm (KL57, Toshiba, Tokyo).

The amount of membrane-bound $TFPB^-$ was determined from the decrease in the free $TFPB^-$ in the supernatant after ultracentrifugation of membrane suspension (90000 × *g*, for 1 h) using the difference of the absorbance between 269 and 277 nm.

Results

$TFPB^-$ induces color change from purple to blue

Fig. 1a shows the effect of varying concentrations of $TFPB^-$ on the spectral change of light-adapted bacteriorhodopsin at neutral pH (pH 7.0 with 10 mM Mops at 25 °C). The spectrum was obtained about 10–20 min after the addition of $TFPB^-$ for each case. As the concentration of $TFPB^-$ increased at the range below 1.0 or 1.5 mM, the absorbance at 475–570 nm decreased with a concomitant increase in the absorbance at broad wavelength region longer than 600 nm in a dose-dependent manner. The difference spectrum with respect to control showed the positive band centered at 640 nm and the negative band centered at 560 nm with an apparent isosbestic point of around 595 nm (data not shown). When the $TFPB^-$ concentration reached to 2 mM, the apparent color turned to blue. This is due to the red shift of maximum wavelength (around 590 nm). This wavelength is somewhat shorter than that of the acid-induced blue membrane whose λ_{max} is 600–608 nm [4–9]. Unfortunately more concentrated $TFPB^-$ converted the blue membrane into the formation of a blue-shifted species whose λ_{max} was around 475 nm. This species is not characterized yet.

At the acidic medium (10 mM citrate/KOH, pH 4.5), on the other hand, addition of a much lower concentration of $TFPB^-$ (0.2 mM) leads to the formation of a red-shifted species whose λ_{max} is 606–608 nm (see Fig. 1b). The color is blue and

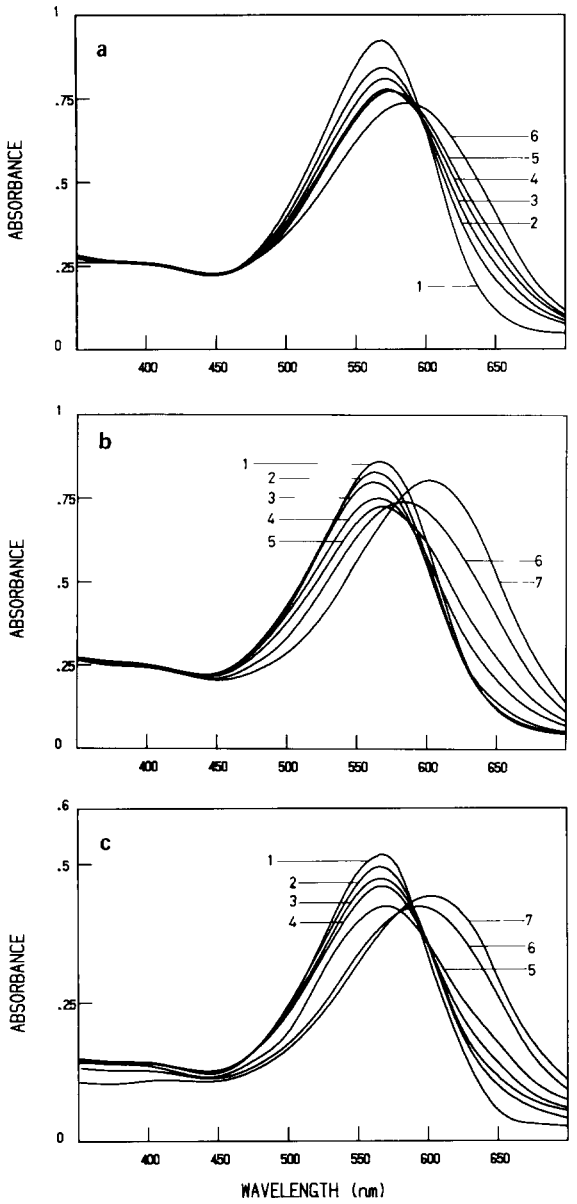

Fig. 1. Spectrum change of bacteriorhodopsin by titration with $TFPB^-$ (a and b) and with HCl (c). (a) Bacteriorhodopsin was suspended in 10 mM Mops (pH 7.0) at 25°C. The spectrum was obtained at 10–20 min after the addition of $TFPB^-$. The concentrations of $TFPB^-$ are 0, 0.5, 0.75, 1.0, 1.5, and 2.0 mM from curve 1 to 6, respectively. (b) Bacteriorhodopsin was suspended in 10 mM citrate buffer (pH 4.5) at 25°C. The concentrations of $TFPB^-$ are 0, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.2 mM, from curve 1 to 7, respectively. (c) Bacteriorhodopsin was suspended in water and small volumes of concentrated HCl were added into a spectrophotometric cuvette which is installed with glass electrode and stirring apparatus. pH values of each curve are 5.5, 3.65, 3.30, 2.88, 2.58, 2.20 and 1.78 from curve 1 to 7, respectively.

λ_{max} is equal to that reported as the blue membrane [4–9]. At concentrations higher than 0.75 or 1 mM, $TFPB^-$ produced the blue-shifted species.

These results in Figs. 1a and 1b are summarized in Fig. 2, where relative increases of the absorbance at 640 nm, A_{640} are plotted. It is clear that effectiveness of $TFPB^-$ becomes higher at lower pH. At pH 7.0, salt enhances the effectiveness of $TFPB^-$. But, at pH 4.5, curves obtained in the presence of 100 mM, 500 mM and 2 M NaCl are almost the identical to those in the absence of salt when the $TFPB^-$ concentrations are low (in the figure, only the data of 2 M NaCl are shown), indicating that there is no salt effect at pH 4.5. At several hundreds of μM of $TFPB^-$, however, the relative value of A_{640} becomes smaller than that in the absence of salts, which is due to the formation of the blue-shifted species.

The red-shifted species are stable for a few hours after addition of $TFPB^-$, but gradual loss of absorbance at 570–610 nm was observed during longer incubation due to the formation of the blue-shifted species. Illumination accelerated its formation. When $TFPB^-$-absorbed membrane was

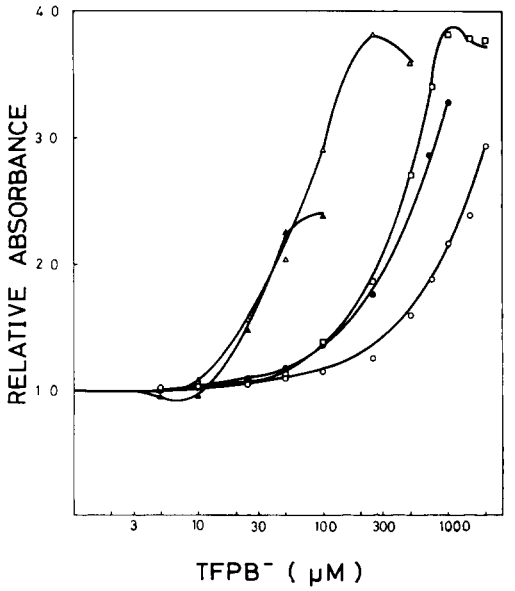

Fig. 2. Relative increase in the absorbance at 640 nm is plotted against the $TFPB^-$ concentration (log scale) ○, pH 7.0 of 10 mM Mops, ●, pH 7.0 of 10 mM Mops and 0.1 M NaCl, □, pH 6.0 of 10 mM Mes, △, pH 4.5 of 10 mM citrate, ▲, pH 4.5 of 10 mM citrate and 2 M NaCl

washed with a large volume of buffer solution free from $TFPB^-$, the purple color was recovered. The blue-shifted species, however, returned only partially to the purple color.

The hydrophobic anion, TPB^- induced a similar spectral change. K^+ in the solution, however, produced precipitation with TPB^- and the accurate spectroscopical measurements have not been done. Di-2-ethylhexyl sodium sulfosuccinate also produced the blue membrane, but the stability of the blue membrane produced was poor. It was founded that picrate anion, a typical lipophilic anion, did not induce a color change up to 10 mM (which was due to poor binding to bacteriorhodopsin).

Flash-photolysis and CD spectra of blue membrane induced by $TFPB^-$

Fig. 3 shows the flash-induced difference spectrum of the $TFPB^-$ induced-blue membrane (pH 4.5) at 1.5 ms after the flash as well as that of the control. The depletion maximum in the spectra is located around 630 nm. The M-intermediate was not observed and an intermediate was observed whose λ_{max} in the difference spectra was approx. 510–520 nm. Mowery et al. [16] obtained flash-induced difference spectra of the acid-induced blue membrane and found a 510 nm intermediate (see also Ref. 17). The difference spectrum of deionized blue membrane was the same as that of

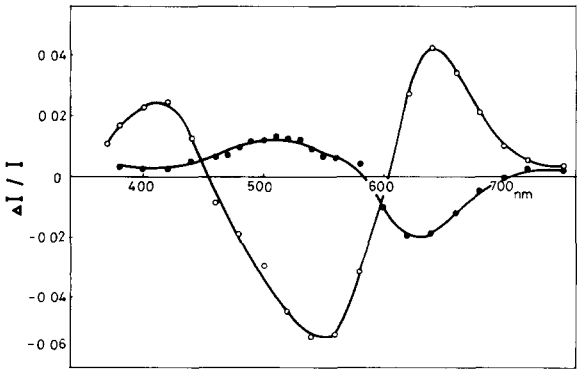

Fig. 3 Flash-induced difference spectrum of the $TFPB^-$-induced blue membrane Bacteriorhodopsin ($A_{570} = 0\,70$) was suspended in 10 mM citrate buffer (pH 4 5) at 25 °C ○, control and ●, 0 2 mM $TFPB^-$ The points were data at 1 5 ms after flash

$TFPB^-$-induced blue membrane (data not shown).

The circular dichroism spectra of the $TFPB^-$-induced blue membrane showed an appreciable decrease and red-shift of the negative band at 300–370 nm as well as a bilobed band centered at 600–610 nm (data not shown). The shift of a bilobed band corresponds to the shift of the absorbance band. These are the same as those obtained with acid-induced or deionized blue membrane reported by previous workers [7,16].

These facts indicate that the $TFPB^-$-induced blue membrane has the same characteristics of chromophore as those induced by acid or deionization.

$TFPB^-$ mimics the action of H^+

Fig. 1c shows the spectral changes of bacteriorhodopsin induced by HCl titration when bacteriorhodopsin is suspended in de-ionized water. Comparison of Fig. 1c with Figs. 1a and 1b indicates that the acid-induced spectrum change is almost the same as that induced by $TFPB^-$ titration. When the HCl titration was done between pH 7 and 2.5, the isosbestic point was 597 nm, which is very close to that shown in Fig. 1a. Acid titration in the presence of $TFPB^-$ showed the formation of blue membrane at higher pH than in the absence of $TFPB^-$ (data not shown, see also Fig. 2).

These facts reveal that $TFPB^-$, a hipophilic anion, has the same effect as H^+, although the sign of its charge is opposite, except for the formation of the blue-shifted species at very high concentrations of $TFPB^-$.

Restoration of purple color by low concentration of hiphophilic cation

The original purple color can be restored by addition of various cations into de-ionized blue membrane [7–9,18,19]. The cation concentrations to the required for almost full restoration were about 10 mM for Na^+ and K^+, and about 0.1 mM for Mg^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Pb^{2+} at 5 μM of protein concentration as reported in [7]. The restoration was also observed by the addition of organic cations. Fig. 4a shows the restoration by addition of TPP^+ to the de-ionized blue membrane. Addition of 0.2–0.4 mM of TPP^+ changed the color from blue to purple. The required con-

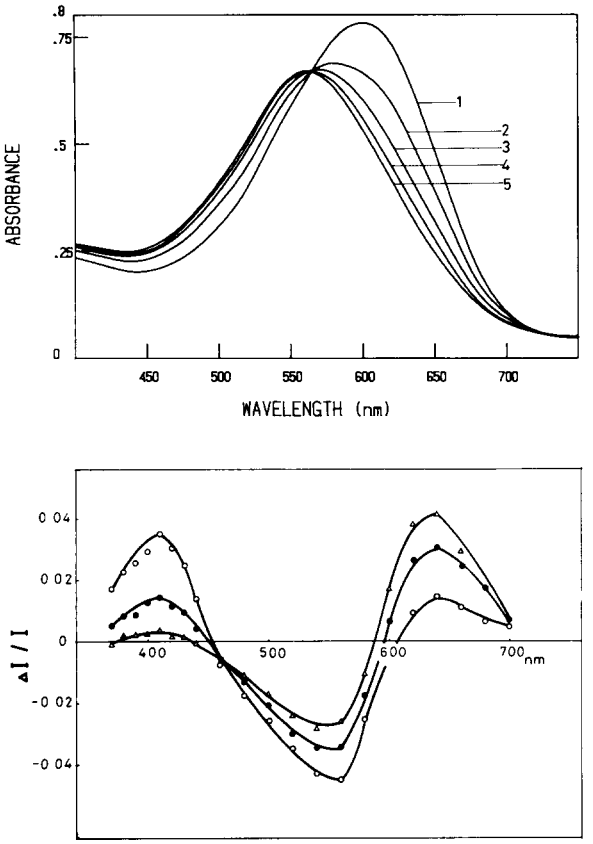

Fig. 4. Restoration of purple membrane from blue membrane by addition of TPP^+, a lipophilic cation The blue membrane was prepared by passing purple membrane through a cation-exchanger column of and the pH of the suspension of the blue membrane was 5 0 (a) The spectral change by addition of TPP^+ The concentrations of TPP^+ were 0, 0 167, 0 334, 0 500 and 0 668 mM, respectively from curve 1 to 5 (b) Recovery of M-intermediate. The concentration of TPP^+ added to the blue membrane was 0 5 mM. The difference spectra are obtained at 1 5 ms (○), 4 8 ms (●) and 10.3 ms (△) after the flash.

centration is lower than that of monovalent cations, but higher than for divalent cations. Flash photolysis data of TPP^+-restored purple membrane (Fig. 4b) showed the formation of the M-intermediate whose half-time of the decay was 2.2 ms (with blue membrane, M is not observed), indicating that the photocycle is restored as well as its color. This is consistent with the finding by Mukohata and his collaborators [20] that various cationic dyes restored the purple color from the deionized blue membrane. Therefore, the following idea seems unlikely: The binding of a specific cation to certain specific binding sites is essential in the color change from blue to the original purple.

Addition of TPP^+ in higher concentrations than 1 mM to purple membrane produced the blue-shifted species (data not shown). The slight blue shift of λ_{max} in Fig. 4 may be related to this. The isosbestic points in Figs. 1 and 4 were similar. The reason for the slight differences is not clear at present. Similar results were obtained with $TPMP^+$ (triphenylmethylphosphonium) or its homologs with varied lengths of the alkyl chains were added (data not shown).

Discussion

Importance of local proton concentration in controlling the color change

The results in the present study are summarized. (1) $TFPB^-$, although it is an anion, mimics the action of a proton. A lower concentration of $TFPB^-$ induces the blue membrane at lower pH values. (2) Addition of TPP^+, a lipophilic cation, to the blue membrane restores the purple color and the formation of the M-intermediate.

It is postulated that the color change from purple to blue is due to the protonation of ionizable groups, maybe carboxyl in the vicinity of chromophore [9]. The findings in the present study are explained by a mechanism that binding of lipophilic anion to the membrane increases the negative surface charge and accumulates H^+ at the surface in a local domain nearby a specific dissociable group. Then, $TFPB^-$ is expected to turn the color of bacteriorhodopsin into blue even in a medium of neutral pH. This mechanism explains that in the acidic pH range, a lower concentration of $TFPB^-$ is required to exert its effect. When lipophilic cations are bound to the blue membrane, the surface potential and local pH are expected to change in the opposite direction to the changes produced by lipophilic anions. This explains the transition from blue to purple membrane by addition of TPP^+. Previous authors have described that addition of anionic detergent [21] or polyelectrolyte [22], or incorporation into vesicles composed of acidic lipids [23] turns bacteriorhodopsin to blue Maeda et al. [24] de-

scribed that acetylated bacteriorhodopsin becomes blue in a higher pH range than the normal bacteriorhodopsin does. The mechanism proposed above also explains these results, at least partially, since these treatments are expected to increase the negative charges in the vicinity of the color controlling surface charges.

A quantitative analysis of the data is attempted by considering the surface potential. Surface potential, ψ (expressed in volt) is expressed as follows [25]

$$q = 11.74(C)^{1/2}\sinh(19.46\psi) \quad (1)$$

where q and C stand for the net surface charge density ($\mu C/cm^2$) of the membrane surface and the molar concentration of (1 · 1) salts in the solution at 25°C. The local H^+ concentration at the surface at ψ in the aqueous phase, $[H^+]_{surface}$ is related to the bulk H^+ concentration, $[H^+]_{bulk}$ as follows:

$$[H^+]_{surface} = [H^+]_{bulk} \exp(-F\psi/RT) \quad (2)$$

$[H^+]_{bulk}$ can be measured with a glass-electrode. $[H^+]_{surface}$ will determine the protonation of the color-regulating carboxyl group(s).

Two types of mechanism are, then, expected to work to change the local proton concentration. One is the effect of nonspecific binding of $TFPB^-$ on the whole membrane surface, which affects membrane surface potential and changes the proton concentration on the whole membrane surface (delocalized charge effect) and the other, the effect of binding of $TFPB^-$ to a special site in the vicinity of ionizable group(s) which controls the absorption spectrum (local charge effect). The discussion below indicates the importance of the latter mechanism

Analysis with delocalized charge effect

The amounts of $TFPB^-$ bound were measured to be $2.2 \cdot 10^{-7}$ mol/mg protein when blue membrane was formed at 0.2 mM of the total $TFPB^-$ concentration at pH 4.5 (Fig. 1b). With use of molecular weight of $2.6 \cdot 10^4$ of bacteriorhodopsin, the change in q, Δq caused by $TFPB^-$ binding is calculated as

Δq = 5.7 negative charges per bacteriorhodopsin molecule

$= -9.1 \cdot 10^{-13}$ μC per bacteriorhodopsin molecule

Assuming that one bacteriorhodopsin molecule has 1140×2 Å^2 in area [7,9], we obtain

$\Delta q = -4.0$ $\mu C/cm^2$

On the other hand, electrophoretic mobility [26] or accumulation of ionic probes [27–29] gave -0.36 to -1.08 $\mu C/cm^2$ as an average surface charge density of purple membranes itself. Then, after binding of $TFPB^-$, the average surface charge is estimated to be -4.4 to -5.1 $\mu C/cm^2$, and the surface potential calculated from Eqn. 1 is expected to -104 to -112 mV at 0.01 M and -14 to -16 mV at 2 M NaCl. The difference in pH between surface and bulk are then 1.8–1.9 at 0.01 M and 0.24–0.27 at 2 M. The C-terminal side of bacteriorhodopsin is expected to have 15–18 negative charges per bacteriorhodopsin, although at pH 4.5, some are considered to be neutralized. These negative charges may prevent $TFPB^-$-binding and hence, an extreme case is that all $TFPB^-$ may bind to the opposite side. If the above calculation is done under this assumption, the differences of pH between surface and bulk are 2.5–2.4 at 0.01 M and 0.42–0.46 at 2M. Therefore, the difference may range 1.8–2.5 at 0.01 M and 0.24–0.46 at 2M.

Fig. 1c indicates that the complete shift of λ_{max} is observed only below pH 2.2. On the other hand, Fig. 1b indicates that 0.2 mM $TFPB^-$ shifts λ_{max} almost completely at a bulk pH of 4.5. The difference of bulk pH values between these two solutions is about 2.3, which is compatible with the expected shift of surface pH before and after the binding of $TFPB^-$ at pH 4.5 of 0.01 M solution However, the $TFPB^-$-induced changes of surface pH in the presence of 2 M NaCl is expected to be only 0.24–0.46 unit, which is too small to explain the observed color-change. At pH 4.5, no salt effect on the spectral change was observed, implying that the surface charge density is zero. Electrophoretic mobility and accumulation of ionic probes, however, showed the presence of negative charges at this pH. This fact is also difficult to be accounted for by the delocalized charge effect.

The amount of binding of $TFPB^-$ at pH 7.0 was $5.7 \cdot 10^{-7}$ mol/mg protein at 2 mM $TFPB^-$. The analysis similar to the above led to the con-

clusion that the results in Figs. 1a and 2 cannot be explained by the mechanism assuming delocalized charge effect, since the difference between local and bulk pH should be as large as 4.8.

The analysis with delocalized charge effects, thus, does not quantitatively explain the data. $TFPB^-$ may bind specifically to the surface in the vicinity of color-regulating group(s) and bound molecules may exert such strong effect especially at higher pH as described in the next section.

Analysis with local charge effect

As expected from Eqn. 1, ψ should depend on the bulk salt concentration C and on the q value. If we assume that the effectiveness of $TFPB^-$ depends on its concentration on the special domain on the membrane surface, it should depend on the value of the surface potential, as is the case for H^+ in Eqn. 2, and on the partition coefficient of $TFPB^-$ to the domain.

$$[TFPB^-]_{surface} = K[TFPB^-]_{bulk} \exp(-F\psi/RT) \quad (3)$$

Here K is the intrinsic partition coefficient (defined at $\psi = 0$) which is assumed to be independent of surface potential value. In Fig. 2, there is a significant salt effect at pH 7.0 but not at pH 4.5 for the effectiveness of $TFPB^-$. The results suggest that the net charge density (and surface potential) on the domain is nearly zero at pH 4.5 but not at pH 7.0. If we assume the surface concentration of $TFPB^-$ to be a main factor in determining the absorption change, we can estimate ψ values which mainly stem from the intrinsic surface charges (Table I) by analyzing the salt effects. The surface pH can be calculated from ψ and the bulk pH as shown in Table I. Values estimated at pH 7.0 are explained if membrane surface has a net charge density of -2.2 to -2.6 $\mu C/cm^2$, in a range similar to those reported in other biological membranes [10]. The mechanism that the penetration of $TFPB^-$ to the site induces the protonation of nearby groups to cause the color change, thus, seems to be rationalized. However, these values of net charge density are larger than those estimated from electrophoretic mobility [26] or accumulation of ionic probes [27–29] in this membrane. In addition, with these latter methods, which give the average surface charge density, a certain net negative charge density is still detected below pH 4.5. These results suggest different distributions of charges on the membrane surface nearby this special color-controlling domain.

TABLE I

ESTIMATION OF SURFACE POTENTIAL, SURFACE pH AND SURFACE CHARGES AT THE $TFPB^-$-BINDING DOMAIN

Concentrations of $TFPB^-$, at which one tenth of the total absorption change was induced at 640 nm, were obtained from Fig. 2. Values of surface potential, ψ, and surface pH (pH_s) created by intrinsic surface charges of the membrane, i.e. under conditions of negligible binding of $TFPB^-$ to the surface, were calculated assuming a Boltzmann distribution of $TFPB^-$ between the surface and the bulk phase, and zero surface potential at pH 4.5 as described in the text. The net surface charge density q is calculated from the ψ values.

pH and ionic conditions		$TFPB^-$ (μM)	ψ (mV)	pH_s	q ($\mu C/cm^2$)
pH 7	10 mM Mops	288	−72	5.7	−2.2
	+0.1 M NaCl	79	−34	5.1	−2.6
pH 6	10 mM Mes	79	−34	5.1	−0.83
pH 4.5	10 mM citrate	15	+5	4.4	0
	+2 M NaCl	18	0	4.5	0

From these facts, it is concluded that the binding of $TFPB^-$ to a special site nearby the chromophore (local charge effect) can explain the results at higher pH range. In addition, it is interesting that picrate, a typical lipophilic anion does not induce the color change, suggesting some requirements of stereochemical configurations to the binding. This is consistent with the presence of a non-polar cavity near the retinal Schiff base of bacteriorhodopsin [30]. The apparent isoelectric point of the local surface covering this hydrophobic domain is estimated to be approx. 4.5 from the present study. Binding of hydrophobic anions in this domain depends on their concentration on this local surface and induces the protonation of the color-controlling group(s) (maybe, carboxyl(s)). The reverse situation may occur for the transition from the blue to purple by addition of hydrophobic cations.

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