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Electrooptical studies on proton-binding and -release of bacteriorhodopsin

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Abstract. Electric field induced pH changes of purple membrane suspensions were investigated in the pH range from 4.1 to 7.6 by measuring the absorbance change of pH indicators. In connection with the photocycle and proton pump ability, three different states of bacteriorhodopsin were used: *(1)* the native purple bacteriorhodopsin (magnesium and calcium ions are bound, the *M* intermediate exists in the photocycle and protons are pumped), *(2)* the cation-depleted blue bacteriorhodopsin (no *M* intermediate), and *(3)* the regenerated purple bacteriorhodopsin which is produced either by raising the pH or by adding magnesium ions (the *M* intermediate exists). In the native purple bacteriorhodopsin there are, at least, two types of proton binding sites: one releases protons and the other takes up protons in the presence of the electric field. On the other hand, blue bacteriorhodopsin and the regenerated purple bacteriorhodopsin (pH increase) show neither proton release nor proton uptake. When magnesium ions are added to the suspensions, the field-induced pH change is observed again. Thus, the stability of proton binding depends strongly on the state of bacteriorhodopsin and differences in proton binding are likely to be related to differences in proton pump activity. Furthermore, it is suggested that the appearance of the *M* intermediate and proton pumping are not necessarily related.

Key words: Bacteriorhodopsin – Blue membrane – Electric-field-induced pH changes – Proton pump – Photocycle

Introduction

Bacteriorhodopsin is the protein in the purple membrane (Oesterhelt and Stoeckenius 1971) which constitutes a part of the plasma membrane of *Halobacterium halobium* and other halophilic bacteria. Upon illumination, bacteriorhodopsin undergoes a photoreaction cycle coupled with a release and uptake of protons (Oesterhelt and Hess 1973; Lozier et al. 1975). It functions as a light-driven proton pump (Oesterhelt and Stoeckenius 1973), establishing a membrane potential which is converted to a chemical potential or used for ATP synthesis under anaerobic conditions.

The colour of the purple membrane is sensitive to the ionic environment of the retinal chromophore. It can be changed from purple to blue by various methods, for example, by acidification (Oesterhelt and Stoeckenius 1971), cation exchange chromatography (Kimura et al. 1984), electrodialysis (Tsuji and Hess 1986, 1987), delipidation (Szundi and Stoeckenius 1987) or addition of a lipophilic anion (Kamo et al. 1987). The blue bacteriorhodopsin undergoes a photocycle which is different from that of the native purple bacteriorhodopsin. Here, the *M* intermediate is not observed during the photoreaction (Kobayashi et al. 1983; Chang et al. 1985; Chronister et al. 1986). The purple colour can be regenerated by addition of cations and/or by alkalinization (Kimura et al. 1984; Chang et al. 1986; Zubov et al. 1986; Tsuji and Hess 1987; Kamo et al. 1987). The regenerated bacteriorhodopsin shows a photocycle qualitatively similar to that of the native bacteriorhodopsin (Chang et al. 1985; Chang et al. 1986).

Since the membrane potential is involved in the photoreaction of bacteriorhodopsin (Michel and Oesterhelt 1976), a perturbation by an externally applied electric field is an efficient method for studying the mechanism of the photocycle and proton pumping. Indeed, such an external field causes a cyclic change in the structure of bacteriorhodopsin both in suspensions (Tsuji and Neumann 1981 a, b; 1983) and in dried films (Borisevitch et al. 1979; Lukashev et al. 1980; Chamorovsky et al. 1983; Maximychev et al. 1984; Tsuji and Hess 1987; Tsuji et al. 1988). The absorbance change in purple membrane suspensions induced by the electric field ($E = 2-20$ kV/cm with a duration ≈ 100 µs) suggests displacements in the orientation of retinal, tyrosine and/or

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tryptophan residues (Tsuji and Neumann 1981 a). It also suggests changes of the micro-environment of aromatic amino acid residues (Tsuji and Neumann 1983) concomitant with pK shifts in, at least, two types of proton binding sites (Tsuji and Neumann 1981 b).

These pK shifts are currently the focus of our interest. In this paper, the field-induced pH changes of native purple membrane suspensions at various pH values are presented and compared with those found for blue membrane and regenerated purple membrane suspensions. The pK shifts of two proton binding sites are discussed within the framework of proton-pump ability.

Materials

Purple membranes were isolated from *Halobacterium halobium* S9 strain according to Oesterhelt and Stoeckenius (1974). Blue membranes were prepared using a cation exchange column AG-50W from Bio-Rad (Kimura et al. 1984). In order to avoid contamination with ions, all experiments were carried out in quartz- or polystyrene vessels. Water was doubly distilled in a quartz container. The concentration of the purple and blue bacteriorhodopsins was determined on the basis of extinction coefficients of $\varepsilon_{568}=63\,000\,M^{-1}\,\mathrm{cm}^{-1}$ (Oesterhelt and Hess 1973) and $\varepsilon_{605}=60\,000\,M^{-1}\,\mathrm{cm}^{-1}$ (Kimura et al. 1984) respectively. The pH of the suspension was adjusted with 0.01 *M* HCl or 0.01 *M* NaOH in the presence of a pH indicator.

Ethyl orange, bromocresol green or *p*-nitrophenol (Kodak) were used as pH indicators. For each indicator the measurable pH range, the pK value, the absorption maximum for the unprotonated form, the wavelength and the concentration used for the following electrooptical measurements are listed in Table 1. Neither the absorption spectrum of bacteriorhodopsin nor that of the pH indicator were altered by adding the indicator to the membrane suspension, suggesting that there are no interactions between these pH indicators and bacteriorhodopsin. Furthermore, no electric field induced absorbance change was observed in pure indicator solutions at the concentrations listed in Table 1. $MgCl_2$ of analytical grade was purchased from Merck and was used without further purification.

Measurements of pH and spectroscopic acid-base titrations

The pH of the suspensions was measured with a glass electrode GK2321C (Radiometer, Copenhagen) connected to a pH meter 26 (Radiometer, Copenhagen). Note that the small amounts of suspensions used for pH measurements were not used for further experiments because of ionic contamination from the pH electrode. The optical titrations of pH indicators were carried out in a thermostatted cell at 293 K with a Cary 219 spectrophotometer (Varian) both in the absence and presence of purple membranes. The presence of purple membranes did not change the titration curves of the pure indicator solutions.

Table 1. The three pH indicators: measurable pH range, pK value, absorption maximum for the unprotonated form, λ_{max}, observation wavelength, λ_{obs}, concentration used for experiments

	ethyl orange	bromocresol green	*p*-nitrophenol
pH range	3.4–4.8	3.8–5.4	5.4–7.9
pK	4.1	4.6	6.5
λ_{max}/nm	475	615	400
λ_{obs}/nm	420	620	410
conc/*M*	5.62×10^{-5}	1.43×10^{-5}	3.60×10^{-5}

Electric field induced pH changes of suspensions were measured with an electric relaxation spectrometer (Eigen and De Maeyer 1963; Schallreuter 1982) developed in our laboratory (Tsuji and Hess 1986). A collimated light beam from a 200 W halogen-mercury lamp passed through a Zeiss monochromator and a Glan-Thompson polarizer. The polarizer was set at the angle where the pure dichroic signal of purple membranes was cancelled out (Tsuji and Neumann 1983).

Electrooptical measurements

A single rectangular electric pulse of 20 kV/cm with a duration of 80 μs was applied to the sample suspensions, unless noted otherwise. The light transmitted through the sample was detected with a photomultiplier, amplified and stored in a transient recorder (Nicolet Model 205-A). The change in the absorbance, ΔA, caused by the electric field was calculated from the transmitted light intensity change, ΔI, according to

$$\Delta A = -\log\left(1+\frac{\Delta I}{I_0}\right), \tag{1}$$

where I_0 is the intensity of the transmitted light in the absence of the electric field. All experiments were carried out at 293 K. The temperature increase due to the Joule heating was negligible (Tsuji and Neumann 1981 a).

Results

(A) Purple bacteriorhodopsin

Figure 1 shows some typical signals for the electric field induced pH changes of purple membrane suspensions. At pH 4.4 (Fig. 1 a, pH indicator: ethyl orange), when the electric field is applied, the intensity of the transmitted light at 420 nm decreases (upper curve of a). After removing the field, the light intensity increases and returns to the initial value (lower curve of a). Since the indicator itself does not show any field-induced optical change at this concentration, and since the polarizer is set at the angle at which no orientational effects are observed, the observed signal is, in fact, due to the pH change of the purple membrane suspension caused by the electric field. Here, a decrease of the light intensity corresponds to an

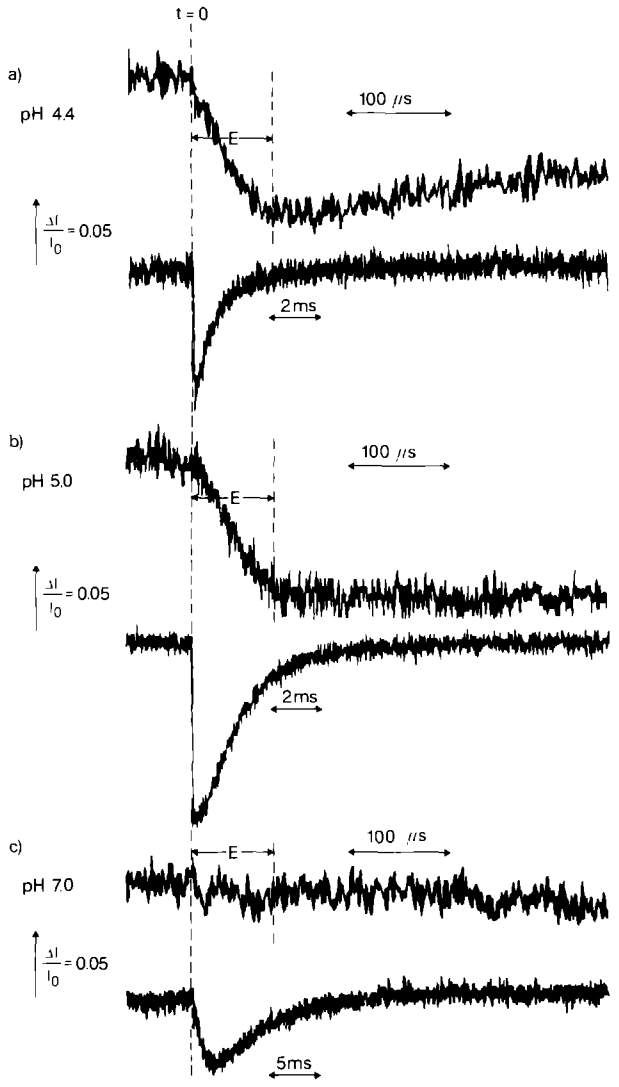

Fig. 1 a–c. Typical optical signals due to the electric field-induced pH changes in native purple membrane suspensions ([bR] = 6.4 $\times 10^{-6} M$): **a** pH 4.4; pH indicator, ethyl orange; $\lambda = 420$ nm, **b** pH 5.2; pH indicator, bromocresol green $\lambda = 620$ nm, and **c** pH 7.0; pH indicator, *p*-nitrophenol; $\lambda = 410$ nm. $E = 20$ kV/cm; pulse duration, 80 µs

increase of the absorption of the unprotonated form of ethyl orange, that is, an increase in pH. (This is also the case for bromocresol green at 620 nm and *p*-nitrophenol at 410 nm).

At pH 5.0 (Fig. 1 b, pH indicator: bromocresol green), the pH of the suspension increases in a sigmoidal manner in the presence of the electric field. The pH is still increasing slightly even after the field is switched off (upper curve of b). At about 0.2 ms after removing the field the pH gradually decreases and returns to the original value (lower curve of b).

At pH 7.0 (Fig. 1 c, pH indicator: *p*-nitrophenol) the observed signal is similar to that obtained previously (Fig. 3 of Tsuji and Neumann (1981 b), (pH indicator: bromothymol blue)). As shown in the upper curve of Fig. 1 c, no pH change is observed during field application. However, after field removal the pH of the suspension increases and then returns to the initial value (lower curve of c). The maximum pH change, ΔpH, after removing the field depends on the pulse duration (the ΔpH increases on increasing pulse length from 20 µs to 100 µs), although no apparent pH change is observed in the presence of the field.

The transient curve at pH 4.4 after conversion from transmitted light intensity to absorbance (see (1)) was analyzed by a single exponential function for the field-on process and by a sum of two exponential functions for the field-off process. The relaxation times were estimated to be 60 µs for the field-on process and 0.5 ms and 2 ms for the field-off processes. Since the pulse duration of 80 µs was not long enough to reach a steady state in the electric field, it was estimated by extrapolation to $t \rightarrow \infty$. From the steady state value and the optical titration curve of ethyl orange (not shown) the number of protons which are taken up by a bacteriorhodopsin molecule was calculated to be 1.3 (± 0.3).

The transient curve at pH 5.0 can be analyzed by two exponential functions, the amplitudes of which have opposite sign. The field-on curve (Fig. 2 a) can be simulated by adding a proton-release process with a time constant of 23 µs and a proton uptake process with a time constant of 61 µs. The steady state value of ΔA, obtained for the proton-uptake process by extrapolation to $t \rightarrow \infty$, is 6 times as large as that for the proton-release process. According to the optical titration curve of bromocresol green (not shown), the number (per bacteriorhodopsin) of protons released and taken up was calculated to be 0.17 and 0.75, respectively. – From now on, we call the site (or sites) from which protons are released in the presence of the electric field "site 1", and the site (or sites) at which protons are taken up "site 2". – When the electric field is switched off, protons are rebound by site 1 (proton re-uptake) and released again from site 2 (proton re-release). The field-off transient curve can be well reconstructed by adding the proton re-uptake process with a relaxation time of 0.2 ms and the proton re-release process with relaxation times of 0.7 ms and 4.3 ms (Fig. 2 b).

At pH 7.0, the optical changes due to the proton release and uptake are cancelled out in the presence of the field (Tsuji and Neumann 1981 b), suggesting that the relaxation times and the absolute steady state values of the absorbance change for these two processes are nearly the same. The field-off curve was decomposed into the proton re-uptake process and proton re-release process with time constants of 2.2 ms and 5.6 ms. From the amplitudes at the moment of field removal, the number (per bacteriorhodopsin) of protons released from site 1 and taken up at site 2 was roughly estimated to be 0.01 and 0.01, respectively. Note that these values are usually smaller than those from the steady state. For shorter field duration both the amplitudes of proton-uptake and -release processes are smaller and consequently the observed signal after removing the field is also smaller.

In such a way we analyzed the transient optical signals in the pH range from 4.1 to 7.6. Figure 3 shows a pH dependence of the number (per bacteriorhodopsin) of protons released and taken up by the electric field pulse. The proton release is caused by the electric field only when the pH exceeds 4.5. The number of the released

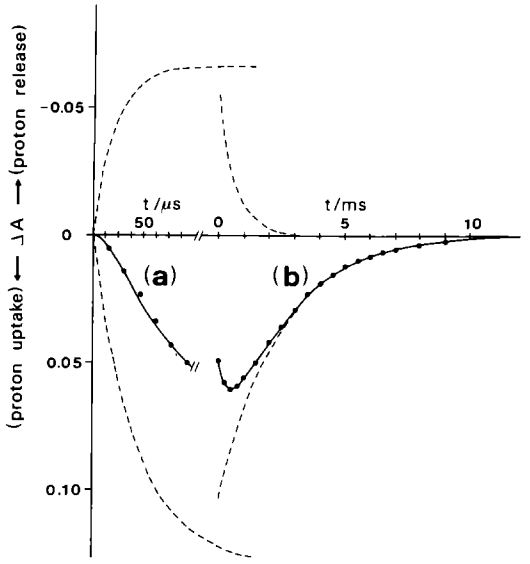

Fig. 2. An example for the kinetic analysis of the electric field induced pH changes. – ● –, measured curves; ——, calculated curves by adding two components (– – –), one for proton release and the other for proton uptake

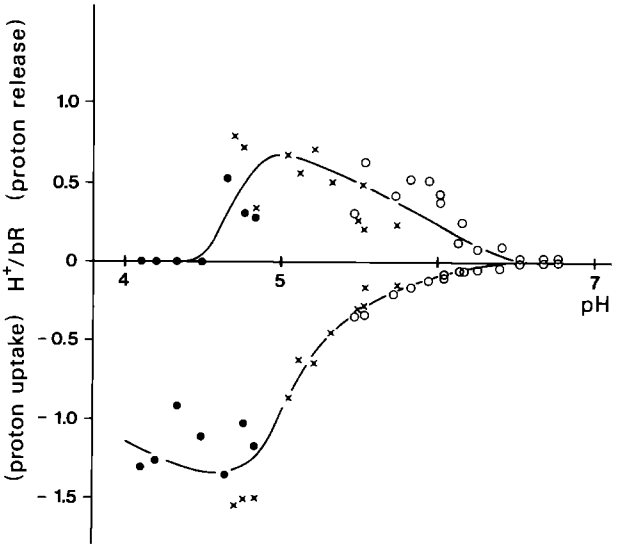

Fig. 3. pH dependence of the number (per bacteriorhodopsin) of protons released and taken up due to the electric field pulse of 20 kV/cm. pH indicators; ethyl orange (●), bromocresol green (×) and *p*-nitrophenol (○)

protons reaches a maximum around pH 5.0 and gradually decreases with increasing pH. On the other hand, the electric field causes proton uptake even at pH 4.1. Around pH 4.6, the number of these protons reaches its maximum value and then decreases with increasing pH. At pH 7.6 no optical change was detected both in the presence and after removing the electric field.

(B) Cation-depleted bacteriorhodopsin

Similar experiments were carried out with cation-depleted bacteriorhodopsin suspensions in the pH range from

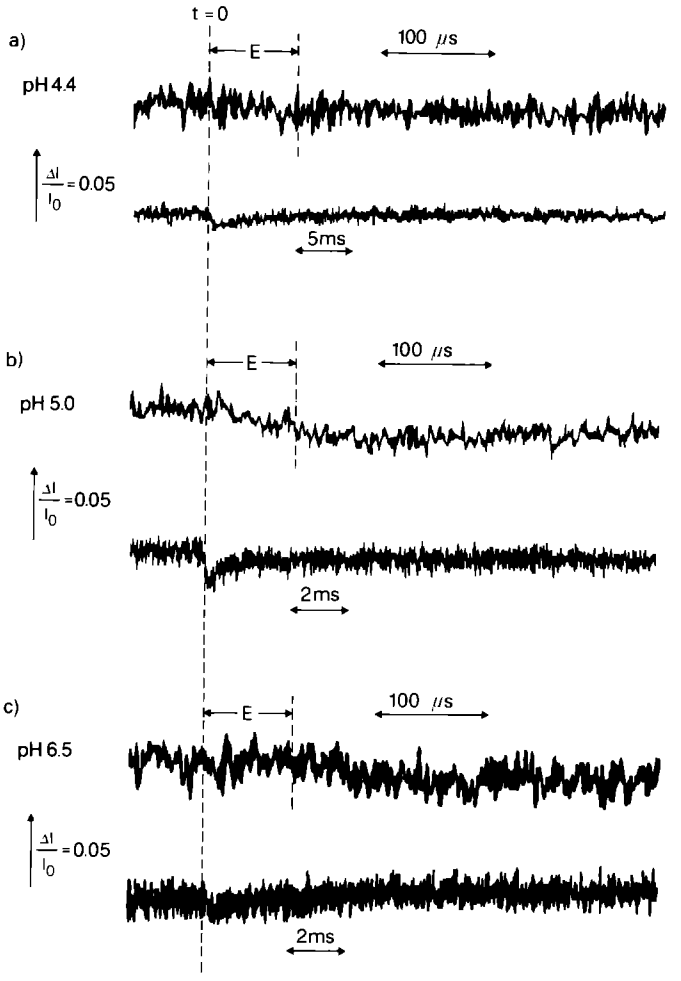

Fig. 4a–c. Typical optical signals due to the electric field-induced pH change in cation-depleted membrane suspensions: **a** pH 4.4, **b** pH 5.0 and **c** pH 6.5. Other experimental conditions, see Fig. 1

4.2 to 6.5. In the pH range from 4.2 to 4.9 the colour of the bacteriorhodopsin remains blue, while at pH 5.0 the purple colour is partially regenerated and at pH 6.5 it is regenerated to >90%. Note that the concentration of sodium ions from NaOH used to increase the pH value is low and NaCl of the same concentration does not affect the blue colour. As shown in Fig. 4, a very slight optical change and consequently a very slight pH change is induced by the electric field in the cation-depleted membrane suspension in the measured pH range.

However, when the cation-depleted membranes are equilibrated with a small amount of magnesium ions, pH changes are again induced by the electric field. Figure 5 shows the optical signals when $[Mg^{2+}]/[bR] \approx 7.5$. The transient curves were analyzed in the same way as for the native purple membrane. At pH 4.3 only the proton uptake (2.1 protons per bacteriorhodopsin) is induced in the electric field. The relaxation time for the field-on process is 40 μs which is faster than that for the native purple membrane at the same pH. The field-off process is characterized by two relaxation times of 0.34 ms and 1.2 ms which are also faster than those for the native purple membrane. At pH 5.0 both the proton-release (0.7 proton/bacteriorhodopsin) and proton-uptake (1.1 proton/

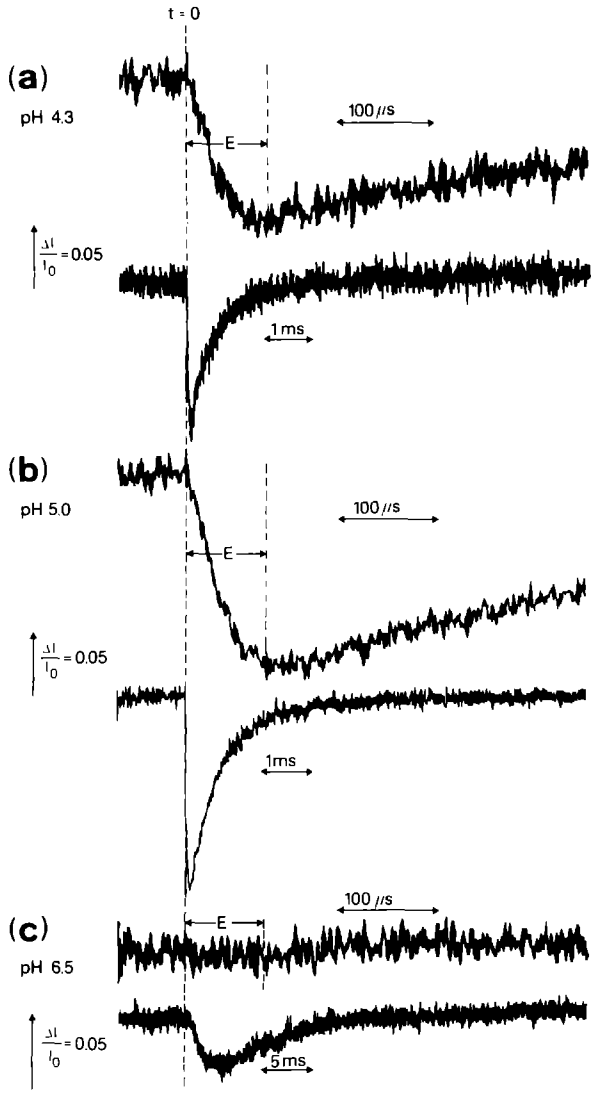

Fig. 5a–c. Optical signals due to the electric field-induced pH change in suspensions of mixtures of cation-depleted membranes and magnesium ions ($[Mg^{2+}]/[bR] \approx 7.5$): **a** pH 4.3, **b** pH 5.0, and **c** pH 6.5. Other experimental conditions, see Fig. 1

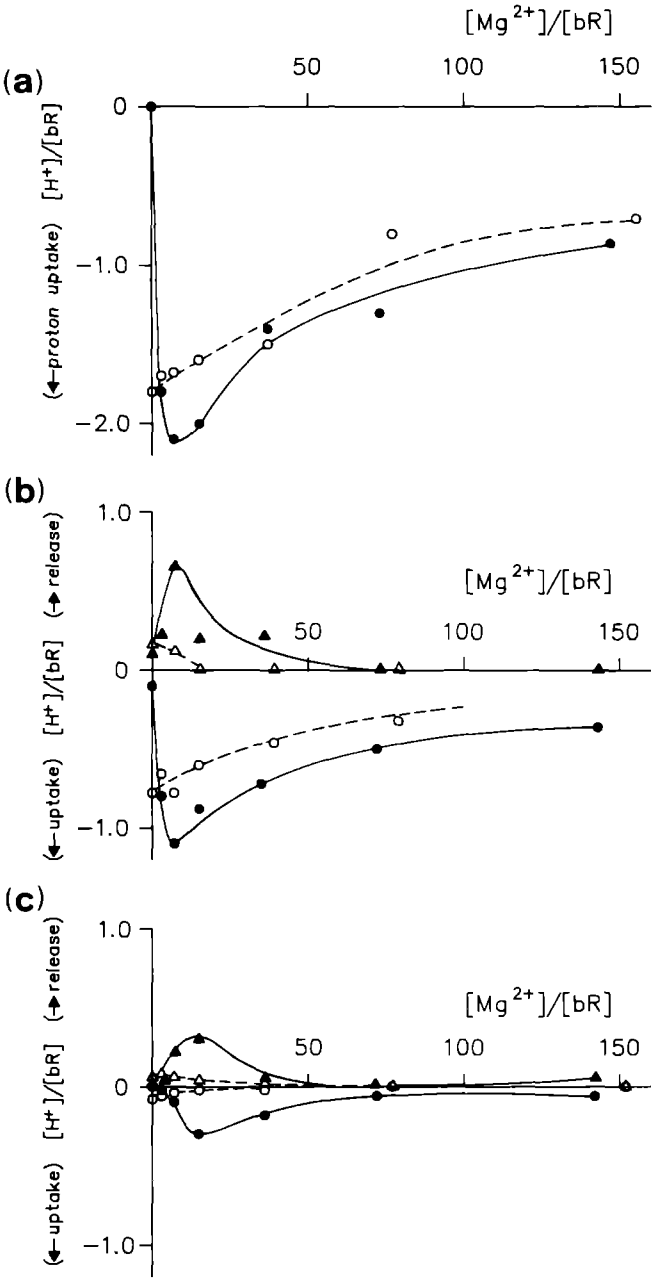

Fig. 6a–c. The number (per bacteriorhodopsin) of protons taken up and released in the presence of an electric field of 20 kV/cm plotted against $[Mg^{2+}]/[bR]$ —, regenerated bR; – – –, native bR: **a** initial pH, 4.3–4.4, **b** initial, pH 5.0 and **c** initial pH, 6.5–7.0

bacteriorhodopsin) with relaxation times of 15 μs and 45 μs, respectively, are induced. The relaxation time of the proton re-uptake process is calculated to be 0.12 ms and those of the proton re-release process are 0.48 ms and 1.2 ms. As is the case at pH 4.3 they are also faster than those for the native purple membrane. At pH 6.5 the transient pH change is observed only after removing the field. However, in contrast to the case of the native purple membrane, the pH change consists of three components: a fast proton re-release process with the relaxation time of 0.1 ms, followed by proton re-uptake and re-release processes with the relaxation times of 1.6 ms and 4.6 ms, respectively. The latter two processes are comparable with two processes in the native purple membrane.

In order to control effects of the ionic strength, the same amount of Mg^{2+} ions was added to the native purple membrane suspensions and the field induced pH changes were measured. In Fig. 6 the number (per bacteriorhodopsin) of protons released and taken up is plotted against the relative magnesium concentration, $[Mg^{2+}]/[bR]$, at three different pH values for both the native purple membranes and the cation-depleted membranes. In the case of the cation-depleted membrane, both the number of protons released and that taken up increase drastically in the range from $[Mg^{2+}]/[bR] = 0$ to $[Mg^{2+}]/[bR] = 7-10$, followed by a relatively gradual decrease. This number 7–10 agrees with the results of Duñach et al. (1987) where they found five high- and medium-affinity sites and five low-affinity sites per bacteriorhodopsin for cation binding. On the other hand, for the native purple membrane it decreases monotonically with increasing relative magnesium concentrations. The latter

result can be explained by a decrease of the effective electric field applied to the membrane with increasing ionic strength.

Discussion

As indicated above, there exist, at least, two types of proton binding sites in the native purple membrane. In the pH range from 4.7 to 7.3 protons are released from site 1 and taken up by site 2 of purple bacteriorhodopsin in the presence of the electric field. However, in the lower pH range from 4.1 to 4.5 only proton uptake is observed.

For the simplest model the following assumptions are made: (1) there are only two proton binding sites and (2) these two sites are independent of each other. Then, two equilibria;

$$bR_1H + Me^+ \rightleftarrows bR_1Me + H^+ \quad (2)$$

$$bR_2Me + H^+ \rightleftarrows bR_2H + Me^+ \quad (3)$$

are established in suspensions, where bR_1 and bR_2 denote the proton binding site 1 and 2, respectively and Me^+ denotes cations which are bound to the native purple membrane. Note that no stoichiometric coefficients are given. The electric field perturbs the corresponding equilibrium states through conformational changes of bacteriorhodopsin (Tsuji and Neumann 1981 b; 1983), shifting them to the right hand side. When the concentration of protons is not so high (pH 4.7–pH 7.3), both equilibrium shifts can be observed. On the other hand, at the higher proton concentration the equilibrium shift in (2) due to the electric field is cancelled out by the equilibrium shift due to the high proton concentration. Therefore, only proton uptake is observed. As can be seen in Fig. 3, the pH dependence of the number of protons released or taken up indicates that the electric field of 20 kV/cm decreases the pK value of site 1 from 5.0 to 4.6 and increases that of site 2 from 4.5 to 5.5. Note that these values are macroscopic averages of several proton binding sites. It is not clear why the number of protons taken up and released decreases on further increasing the pH. It would, of course, be interesting to carry out the same kind of experiments in the pH range above 7.6, especially pH around 10 where tyrosine residues are titrated. However, since no buffer can be used for our purpose, the measurements in the higher pH range have not yet been successful.

Let us compare our results with light-induced proton pumping. It has been established that in light-driven proton pumping there are also two types of proton binding sites; protons are released from one site (the extracellular surface) and taken up by another site (the cytoplasmic surface) (Lozier et al. 1976; Renard and Delmelle 1985). According to Takeuchi et al. (1981), the pK value for the proton-release site is lowered and that for the proton-uptake site is raised upon illumination. Furthermore, in the neutral pH region, the pH of purple membrane suspensions first decreases after a light flash, and then returns to the initial pH (Oesterhelt and Hess 1973; Govindjee et al. 1980). This transient signal is similar to the one shown in Fig. 1 c – just the sign is opposite, because the sequence of pH-increase and -decrease occurs the other way round. This opposite sign in the series of the pH changes suggests that the increase in the membrane potential due to light-driven proton pumping exerts a negative feedback via electric field induced pK changes of two kinds of proton binding sites, probably in order to avoid breakdown of the membrane.

Taking into account that some carboxyl groups of the protein are protonated in the blue bacteriorhodopsin (Gerwert et al. 1987), the following equilibrium is established in the suspension of the cation-depleted membrane at the low pH (<4.9):

$$b-bR_iH \rightleftarrows b-bR_i^- + H^+ \quad (4)$$

where $b-bR_i$ denotes the i^{th} proton binding site of the blue bactriorhodopsin. The electric field of 20 kV/cm is not sufficient for this equilibrium shift (Tsuji and Hess 1988). When a small amount of NaOH is added to the cation-depleted membrane suspension, the equilibrium is shifted to the right hand side and the purple colour is regenerated. However, as long as no extra metal ions are added, neither proton-release nor -uptake is caused by the electric field of 20 kV/cm. This suggests that the colour sensitive site is not necessarily responsible for the proton-release and -uptake.

When Mg^{2+} ions are added to the cation-depleted membrane suspension, a bacteriorhodopsin-magnesium complex, bRMg, is formed, followed by conformational changes through some intermediates, ΣI, reaching a regenerated purple state, as has been described before (Zubov et al. 1986). The reaction model is given as

$$b-bR^- + Mg^{2+} \rightleftarrows bRMg \rightleftarrows \Sigma I \rightleftarrows bR\,. \quad (5)$$

The purple state is similar to the native purple state, but, not the same. In contrast to the case of the cation-depleted bacteriorhodopsin, equilibrium shifts similar to (2) and (3) can be induced by the electric field of 20 kV/cm.

Thus, there are some differences in the stability of the proton binding states of bacteriorhodopsin. The proton binding sites in the cation-depleted bacteriorhodopsin are so stable that the electric field of 20 kV/cm can cause neither proton release nor uptake, even if the purple colour is regenerated by raising the pH. In contrast, in the presence of Mg^{2+} ions, where bacteriorhodopsin forms a complex with Mg^{2+} ions upon releasing protons (Tsuji and Hess 1988), proton- and magnesium-binding to bacteriorhodopsin become weaker. Proton binding in the native purple bacteriorhodopsin is also not so strong, probably because some Mg^{2+} and Ca^{2+} ions are bound in the native state (Chang et al. 1985).

The effects of the light and of the electric field on the regenerated purple membrane (by magnesium ions) are nearly the same as those on the native purple membrane; the *M* intermediate (a relatively stable intermediate with the deprotonated Schiff base) exists in the photocycle, protons are pumped during illumination, and probably therefore the electric field causes the proton-release and -uptake through a negative feedback.

With respect to the proton pumping of the cation-depleted blue bacteriorhodopsin no experiments have yet

been done. However, since no *M* intermediate is observed in the photocycle (Kobayashi et al. 1983; Chang et al. 1985; Chronister et al. 1986), it has been believed that no protons are pumped. The stability of the proton binding in the electric field also supports this idea.

Along this line the *M* intermediate and the proton pump ability have been considered to be two phenomena with one origin. However, this may not be always the case. The regenerated purple membrane (by raising pH) undergoes practically the same photocycle as the native purple membrane (Chang et al. 1985, 1986). Nevertheless, no proton-release and -uptake were observed in the electric field, suggesting that protons may not be pumped under illumination. (Similar to the case of the blue bacteriorhodopsin, unfortunately direct measurements of proton pumping in reconstituted vesicles have not been successful without any additional cations.) We would like to emphasize here only the difference in the electric field between the regenerated purple membranes produced by raising pH and those produced by adding Mg^{2+} ions and suggest a case where the *M* intermediate and the proton pump ability are not related.

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