Buffer Effects on Electric Signals of Light-Excited Bacteriorhodopsin

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ABSTRACT Buffers change the electric signals of light-excited bacteriorhodopsin molecules in purple membrane if their concentration and the pH of the low-salt solution are properly selected. "Positive" buffers produce a positive component, and "negative" buffers a negative component in addition to the signals due to proton pumping. Measurement of the buffer effects in the presence of glycyl-glycine or bis-tris propane revealed an increase of ~2 and a change of sign and a decrease to ~ −0.5 in the translocated charge in these cases, respectively. These factors do not depend on temperature. The Arrhenius parameters established from the evaluation of the kinetics indicate activation enthalpies of 35–40 kJ/mol and negative activation entropies for the additional signals. These values agree with those found by surface-bound pH-sensitive probes in the search of the timing of proton release and uptake. The electric signals were also measured in the case of D_2O solutions with similar results, except for the increased lifetimes. We offer a unified explanation for the data obtained with surface-bound probes and electric signals based on the clusters at extracellular and cytoplasmic sites of bacteriorhodopsin participating in proton release and uptake.

INTRODUCTION

Light-excited bacteriorhodopsin (bR) molecules translocate protons through the plasma membrane of *Halobacterium salinarum* from the cell to the external medium. This is a light-energy transduction phenomenon, and because of its importance, a large range of methods is applied to elucidate its molecular mechanism (Keszthelyi, 1988). This paper concentrates on the electric events associated with proton translocation through bR and compares these signals with data on proton release and uptake.

In different buffered solutions such components of the electric signal were found that did not have counterparts among the light absorption signals (Liu et al., 1991). There were two classes of buffers: "positive " buffers, which produced additional positive electric signals, and "negative" buffers, which produced additional negative electric signals. Signal directions are assigned in relation to the direction of proton translocation: a positive signal means charge motion coinciding with it. The time course of these signals followed, more or less, the so-called B2 component of the current (classified as corresponding to the L → M transition, i.e., the deprotonation of the Schiff base; Keszthelyi and Ormos, 1989). The "negative " buffer effect has been observed earlier and discussed in detail (Tóth-Boconádi et al., 1986; Marinetti, 1987; Dér et al., 1988).

To explain the buffer effect associated with the B2 component Liu et al. assumed that the protons emitted into the solution during M formation protonated the buffer molecules, which were repelled or attracted ("positive" buffers or "negative" buffers, respectively) by the negative charge remaining on the membrane surface after proton release (Liu et al., 1991). Recently, Saga et al. (1999) reported on the effect of buffers on the photoelectrochemical response of bR.

The timing of proton release after light excitation of bR was measured via pH-sensitive dyes in the solution. The data demonstrated that the emitted protons produced the absorption changes in dyes much later than the L → M transition, whereas buffers added to the solution could speed up the absorption changes. In this way, the rise time of the signal approached the lifetime of the L → M transition (Drachev et al., 1984; Grzesiek and Dencher, 1986; Váró and Lanyi, 1991). On the basis of the work of Engasser and Horvath (1973) it was assumed that the protons were really emitted during the L → M transition, but it took time to reach the dye molecules dissolved in the solution. The added buffer molecules accelerated the proton conduction to the dye molecules.

Buffer molecules were not necessary for a fast response to presume proton emission when pH-sensitive probes were bound to the external surface of bR molecules (Heberle and Dencher, 1990, 1992). The rise time of the absorption change of these probes was close to the time constants of M formation (at high temperature to its second, and at low temperature to its third component). Its temperature dependence, however, corresponded to an activation enthalpy quite different from those of the two mentioned formations. It had a decay component too, with a lifetime of ~700 μs (at around room temperature), close to the rise time of the absorption change of a pH-sensitive water-soluble dye, pyranine. The interpretation of these findings was that the protons appeared at the surface of the membrane as fast as the B2 component of the current (Liu et al., 1991) and moved via diffusion in the interfacial layer for ~700 μs before they were released into the solution. This is the time when they were detected by the proton-sensitive dye pyranine.

The assumption of fast proton release involves problems: Fourier transform infrared data demonstrate that the amino

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acid D85 is protonated from the Schiff base during the L → M decay, and the proton stays on it during the lifetime of the M components (Gerwert et al., 1990). Therefore, the Schiff base proton cannot be the released proton. In a different approach to the problem, Zimányi et al. (1992) postulated that the released proton originates from an unknown (XH) residue, the deprotonation of which depends greatly on the pH of the solution. Recently, evidence was published that Glu^{194} or Glu^{204} may be that residue (Brown et al., 1995; Balashov et al., 1997; Dioumaev et al., 1998) and that water molecules take part in the process (Rammselsberg et al., 1998). We may call this moiety of bR a release cluster at the extracellular (EC) side, similar to the proton uptake cluster at the cytoplasmic (CP) side, suggested by Brown et al. (1994) and Checover et al. (1997).

We set out to acquire more information on this important problem by studying the effects of buffers on the electric signals. We therefore selected a "positive" buffer (glycyl-glycine, Gly-Gly) and a "negative" buffer (bis-tris propane, BTP) from the 31 buffers studied by Liu et al. (1991) and, in addition to the B2, measured all other components of the electric signals. We also determined the temperature dependencies of the rate constants.

The total area of the electric signal was found to be about twice as large in the case of the solution containing Gly-Gly than without it and negative in the case of the BTP-containing solution, independently of the temperature at the selected buffer concentrations and pH. The activation enthalpies of the rise and decay of the electric signals of the additional B2 component due to the buffers were similar to the activation enthalpies determined for surface-bound pH indicator (Heberle and Dencher, 1992; Heberle et al., 1994; Alexiev et al., 1995; Cao et al., 1995). These activation enthalpies differed from the values measured for the intermediates of the bR photocycle and from those characterizing the temperature dependence of diffusion processes and of conductivities of different ionic solutions. We assumed that the two phenomena have a common origin; therefore we elaborated a hypothesis based on the above-mentioned release and uptake clusters to explain the results obtained with the surface-bound pH indicator and recorded the electric signals in the presence of buffers.

MATERIALS AND METHODS

Purple membrane (pm) prepared from *Halobacterium salinarum* strain R1M1 or ET 1001 by the usual method (Oesterhelt and Stoeckenius, 1974) was oriented and immobilized in polyacrylamide gel as described by Dér et al. (1985). Slabs measuring 1.6 × 1.6 × 0.18 cm were cut and soaked in the solution (~100 cm^3) with given parameters for overnight at least and placed in the same solution into cuvettes. The temperature of the sample was maintained by circulating water and measured with a thermocouple immersed in the solution. The samples were illuminated with a dye laser (Rhodamin 6G) driven by an excimer laser (EMG 101 MSC; Lambda Physik, Göttingen, Germany) of 4–5 mJ energy, and by quasicontinuous illumination for 1–2 s with a 200-W tungsten lamp. Platinized Pt electrodes immersed in the solution picked up the electric signals, which were amplified with a homemade current amplifier, based on a Burr-Brown 3554 operational amplifier, and digitized with a Thurlby DSA-524 computer-controlled transient recorder (Thurlby Electronics, Huntington, UK) (after appropriate filtering in the millisecond time range).

Light from the same lamp after passage through an interference filter of 400 nm served to measure the rise and decay of the M intermediate.

The data from the digitizer were fed into a personal computer, and time constants were evaluated with the SPSERV program, written and kindly provided by Cs. Bagyinka from our Institute.

The amplitudes of the electric signals were normalized to the first component, negative compared to the direction of proton translocation. This was necessary because the pairwise measurements, at a given temperature, were performed on different cuts of the gel, which might have had a somewhat different level of orientation. This procedure involves the plausible assumption that all of the components of the electric signal are proportional to the first signal.

RESULTS

Glycyl-glycine and bis-tris propane buffers in H_2O

The B2 components of the electric signals measured in the case of pm in 50 μM $CaCl_2$ and 5 mM Gly-Gly and in 1 mM BTP at pH 7.5 were practically the same as those found by Liu et al. (1991). The curves registered at different temperatures (between 6 and 31°C) were normalized to the first negative component at every temperature, and the differences (buffered − unbuffered) were calculated. Fig. 1 depicts the measured electric signals and Fig. 2 the difference curves at 20°C for both buffers. The differences have fast rise and slow decay components in the microsecond time domain and a longer living component for BTP buffer. The contribution in the millisecond range is negligible for Gly-Gly buffer. Figs. 3 and 4 present the temperature dependence of the rate constants.

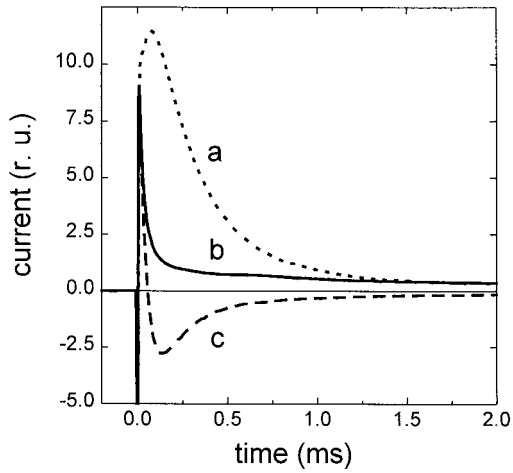

FIGURE 1 Electric signals measured in the presence of Gly-Gly (*curve a*) and BTP (*curve c*) buffers and without buffers (*curve b*) in H_2O. Buffer concentration 5 mM Gly-Gly, 1 mM BTP, respectively, pH 7.5, temperature 20°C. Both bathing solutions contained 50 μM $CaCl_2$.

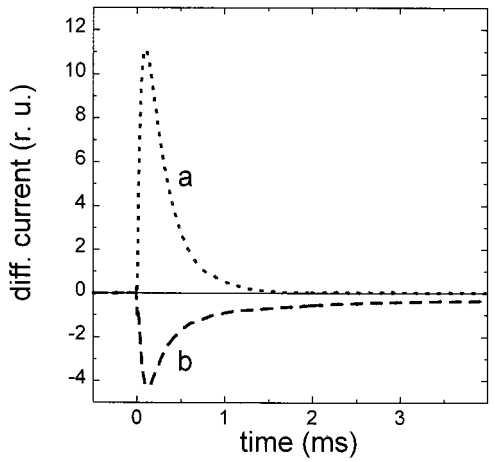

FIGURE 2 Difference of the signals measured in solution with 5 mM Gly-Gly (*curve a*) and 1 mM BTP buffer (*curve b*) and without it. Parameters as in Fig. 1.

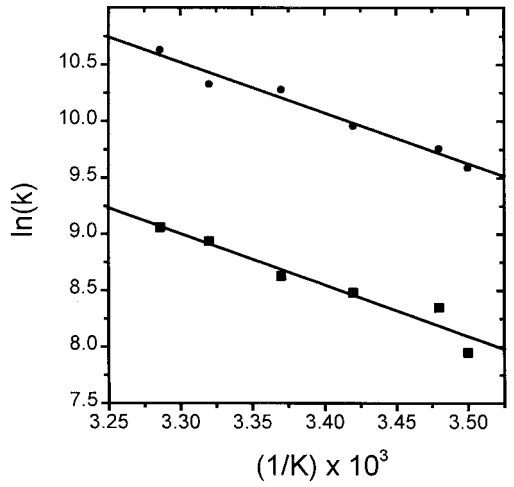

FIGURE 3 Arrhenius plot of the rate constants *k* of the difference curves for Gly-Gly buffer. ●, rise; ■, decay.

The area of the electric signal A (amplitude $\times$ time) is well known to be proportional to the number N of charges Q translocated to a distance d (Keszthelyi and Ormos, 1989). Fig. 5 depicts the time dependence of the integrated areas of the signals from the two samples (buffered versus unbuffered) for Gly-Gly buffer. The ratio is $R \approx 2$. The same ratio resulted when the samples were illuminated for a longer time with quasicontinuous light. Fig. 6 illustrates the quasicontinuous currents at 20°C. An increase by a factor of $R \cong 2$ was observed in this case, too. Fig. 7 shows the

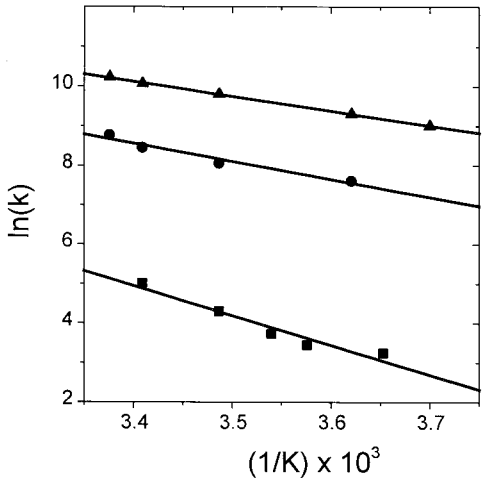

FIGURE 4 Arrhenius plot of the rate constant *k* of the difference curves for BTP buffer. ▲, rise; ●, fast decay; ■, slow decay.

temperature dependence of the ratios of the areas produced in a single cycle and of the amplitudes of the currents produced by quasicontinuous illumination. The values are near each other and reveal a constant ratio of 1.9–2. Two additional series gave similar data. The mean value of the increase measured in single cycles and quasicontinuous experiments, in three series for Gly-Gly buffer, is $R_G = 1.98 \pm 0.04$.

The integrated area and the magnitude of the quasicontinuous current proved to be negative and temperature-independent for the BTP buffer too (not shown). The mean value of the ratios of the areas relative to those for the

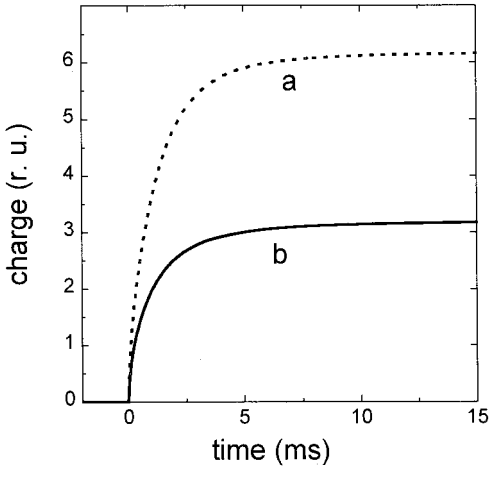

FIGURE 5 Time dependence of the integral of the electric signals in the case of laser-flash excitation. *Curve a*, with Gly-Gly buffer; *curve b*, without it.

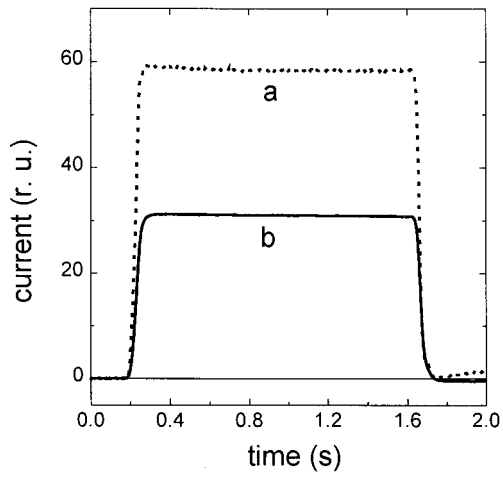

FIGURE 6 Currents measured in the case of quasicontinuous illumination. *Curve a*, with Gly-Gly buffer; *curve b*, without it.

buffer-free solution is $R_B = -0.55 \pm 0.05$, from three independent determinations.

The optical data concerning the rise and decay of the M intermediate do not differ between the buffered and unbuffered samples. The temperature dependence of the lifetimes of the components (not shown) differ greatly from those calculated from the difference curves.

Glycyl-glycine and bis-tris propane buffers in D_2O

The measurements were repeated in D_2O solutions. In Fig. 8 we compare the electric signals measured in D_2O solution with the signals measured in H_2O solution in the presence of the Gly-Gly and BTP buffers in the millisecond time range. The forms of the signals are similar; the time courses, however, are different. The ratios of the lifetimes of the rise and decay of the difference curves in D_2O and H_2O at 23°C are 3.6 for Gly-Gly buffer and 6.8 for BTP buffer. Similar data were obtained by Cao et al. (1995) for the time behavior of the proton-sensitive probe at the EC surface. In the case of diffusion these ratios should be ~1.4. The temperature dependence of the rate constants was also registered (not shown). In D_2O the mean values of the ratios of the time-integrated areas of the electric signals measured in buffered and buffer-free solutions are $R_G = 1.8 \pm 0.2$ and $R_B = -0.23 \pm 0.02$. While R_G values are similar for H_2O and D_2O solutions, the R_B value for D_2O is about half of that for H_2O. This difference may be attributed to the long-lived component of the current.

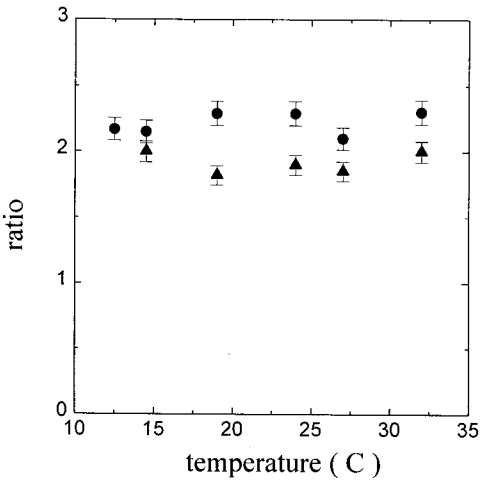

FIGURE 7 Temperature dependence of the ratio of the currents with Gly-Gly buffer and without it. ▲, laser flash excitation; ●, quasicontinuous illumination.

DISCUSSION

The observed phenomena may be summarized in seven points:

1. The "positive" buffer Gly-Gly changes the area of the electric signal and the size of the quasicontinuous current, increasing them with a factor of $R_G \approx 2$; the "negative" buffer BTP inverts and decreases them with $R_B = -0.55$ at the given pH, concentration of buffers, and $CaCl_2$. This latter effect was earlier observed and discussed in the case of tetramethylethylenediamine (TEMED) buffer (Tóth-Boconádi et al., 1986; Marinetti, 1987; Dér et al., 1988). With TEMED, in the right concentration and pH, ratios of $R_T = -1$ were obtained identically for single cycles and quasicontinuous illuminations.

2. The rate constants of the rise and decay of the additional B2 components do not have known counterparts in the bR photocycle. The activation enthalpies and entropies calculated from the temperature dependencies are presented in Table 1. The following equation was used for the calculations:

$$k = 10^{13}\, e^{1/R(\Delta S - \Delta H/T)} \quad (1)$$

where $k = 1/\tau$ is the rate constant, ΔH and ΔS are the activation enthalpy and entropy, respectively, and R and T are the Boltzmann constant and temperature. The activation enthalpy values differ from the values found in the bR photocycle (in the range of 60–80 kJ/mol; Váró and Lanyi, 1991; Cao et al., 1995) and from the values of the temperature dependence of the conductivity of ionic solutions and of the diffusion processes (15–20 kJ/mol). The negative values of the activation entropies demonstrate that the underlying phenomena proceed in the direction of order.

3. The "positive" (Gly-Gly) and "negative" (BTP) buffers differ in their influence on the electric signals. While the "positive " buffer does not influence the millisecond component (practically the entire increase in the area originates

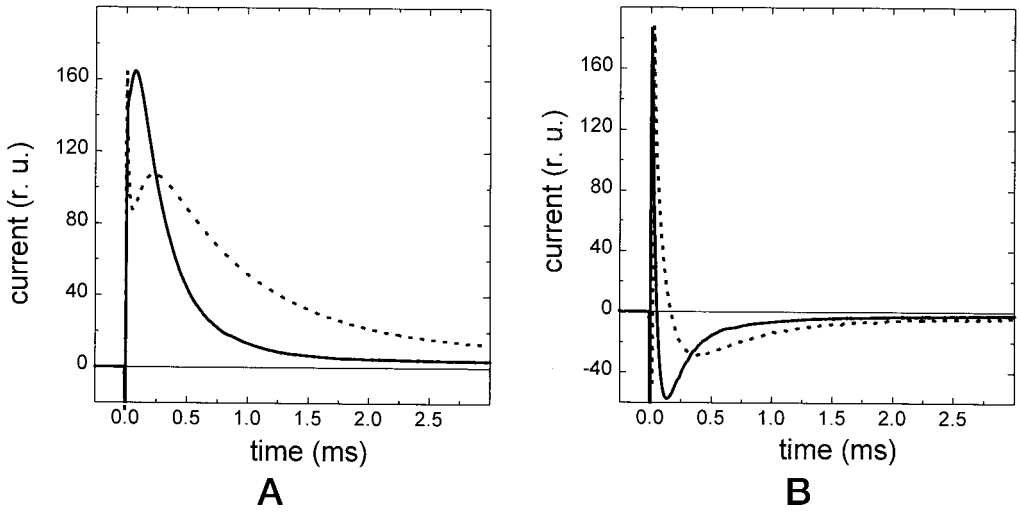

FIGURE 8 Comparison of the electric signals in H_2O (——) and D_2O (·····) solutions in the millisecond time range. (*A*) Gly-Gly buffer. (*B*) BTP buffer. The pH was set to 7.5 in H_2O and 7.9 in D_2O solution to ensure the comparability. Conditions: 50 μM $CaCl_2$, 23°C

from the additional B2 component), the "negative " buffer also causes a change in the millisecond component. To see the tendency only, a single rate constant was fitted to the negative component in the millisecond range, and activation enthalpies and entropies were calculated. These values are also reported in Table 1. They are in the usual range of the values of the bR photocycle. The area of the microsecond component of the additional charge motion is about one-half of the total area.

4. The buffers exert a remarkable influence on the photocurrents corresponding to the single cycles and the quasicontinuous currents in quasicontinuous illumination. The reason for this is a real charge translocation in addition to the charge translocation and real current in addition to the regular quasicontinuous current due to the photocycle. The data show that the transported currents do not flow back, at least in the time of registration (seconds; see Fig. 6). Therefore explanations based on one-sided charge motions, suggested by Marinetti (1987), are not sufficient, as pointed out earlier (Dér et al., 1988).

5. The buffer effects depend on pH and are absent in solutions containing higher salt concentrations (Tóth-Boconádi et al., 1986; Liu et al., 1991).

6. The buffers essentially do not influence the photocycle.

TABLE 1 Activation enthalpies (ΔH, kJ/mol) and entropies (ΔS, kJ/mol degree) for the rise and decay of the difference curve for Gly-Gly and BTP buffers in the μs domain and for the decay of the ms component in the BTP buffer

	Electric				Fluorescein	
	Gly-Gly		BTP			
Process	ΔH	ΔS	ΔH	ΔS	ΔH	ΔS
Rise						
(H_2O)	36.7 ± 1.4	−0.041 ± 0.005	35.7 ± 1.0	−0.040 ± 0.004	35*	−0.040*
					46[†l]	−0.01[†l]
					30[†h]	−0.067[†h]
					37[‡]	−0.048[‡]
(D_2O)	28 ± 3	−0.08 ± 0.01	36 ± 6	−0.053 ± 0.008		
Decay						
(H_2O)	42.3 ± 1.8	−0.031 ± 0.008	41.5 ± 1.6	−0.033 ± 0.005	38*	−0.055*
(D_2O)	40 ± 2	−0.052 ± 0.014	39 ± 5	−0.066 ± 0.010		
Decay of ms component						
(H_2O)			68.0 ± 3.3	+0.024 ± 0.014		
(D_2O)			67 ± 8	+0.010 ± 0.005		

For comparison, the values of ΔH and ΔS (calculated by us) of the fluorescein probe signals are also presented.
*Heberle and Dencher (1992).
[†]Alexiev et al. (1995).
[‡]Cao et al. (1995).
l, low temperature; h, high temperature.

7. The lifetimes of the difference curves increased more in D_2O solution than expected for a diffusion process. The calculated Arrhenius parameters remained similar to those determined in H_2O solution (Table 1).

The results discussed above are in accord with the results of Liu et al. (1991) and extend them with data involving all of the components of the electric signal and Arrhenius parameters of the transitions. On the other hand, the Arrhenius parameters of the additional B2 components are in accord with the same values of the protonation and deprotonation of the optical probe bound to the external surface of the bR, also listed in Table 1 (Heberle and Dencher, 1992; Alexiev et al., 1995; Cao et al., 1995). Therefore, to understand the meaning of the present experimental information, it is necessary to consider the interpretations given in those papers and investigate the possibility that they could explain our data.

The B2 component was investigated with eight buffer groups of different protonation characteristics, and it was found that a negative additional B2 component appeared in only one group; they were positive in all of the others (Liu et al., 1991). The model presented as an explanation involved the motion of protonated buffers in the electric field due to the negative charge remaining on the surface of pm after proton release. This model cannot explain the sign of the additional B2 component in all buffer groups. Furthermore, this model, assuming one-sided charge motion, cannot explain the changes in magnitude of the transported charge after flash excitation or of the current during quasi-continuous illumination, as pointed out in 4.

The interpretation of the results obtained with the optical monitors of the proton translocation (Heberle and Dencher, 1992) likewise runs into difficulties. First, the protonation of the probe is assigned to intraproteinous transfer (Heberle et al., 1994) and the delay in deprotonation to dwelling of protons in the surface layer, but more arguments are not given to explain the measured activation enthalpies and the negative activation entropies (not evaluated in this paper). The diffusion of protons has an activation enthalpy of ~18 kJ/mol, and diffusion is not an ordering effect with negative activation entropy. Second, it assumes proton motion parallel to the membrane, i.e., the protons dwell in the surface layer, while the electric current, with coinciding Arrhenius parameters, reflects perpendicular motion.

We may consider the study on the proton migration along the membrane surfaces (Heberle et al., 1994, Alexiev et al., 1995) as a continuation of the work discussed above. The essential difference was that two independent measurements of protonation were performed. In the first, the probe was at the EC surface of the pm, while in the second, another probe was bound to the CP surface. The probe at the EC surface yielded the same optical data as above, and thus the same criticism applies. The probe at the CP surface demonstrated similar optical changes, but with a shift of ~150 μs at room temperature (Heberle et al., 1994), or practically no shift (Alexiev et al., 1995). The cause of this discrepancy is not known (Heberle, 1999). The activation enthalpy of the shift was ~80 kJ/mol (Heberle et al., 1994), and the entropy, calculated by us, was positive. These parameters are similar to those for the decay of the millisecond component of the buffer effect in the case of BTP buffer (Table 1). The interpretation put forward was that the protons appearing and dwelling at the EC surface were not emitted to the bulk, but went around the pm and protonated the probe at the CP side. This interpretation involves two serious problems: the very high activation enthalpy of the shift, which cannot characterize a type of diffusion offered as an explanation by the authors, and the fact that the onset of protonation at the CP side cannot be accelerated by reducing the size of the pm with fractionation. We may add another problem: the protonation dynamics measurements show that protons dwell on the surface layer of the pm for 40–50 μs (Nachliel et al., 1996), thus for a much shorter time than the measured and assumed dwell time of ~700 μs.

The above-mentioned difficulties in the interpretation of the data in the literature and the new information in our measurements lead us to believe that a new hypothesis is necessary to explain the underlying processes. Naturally, this model should be able to explain the probe and electric signals simultaneously.

Recent studies with mutants assume that the proton release and uptake may occur in clusters at the EC side (Glu^{194}, Glu^{204}, and water molecules; Zimányi et al., 1992; Brown et al., 1995; Rammselsberg et al., 1998; Balashov et al., 1997; Dioumaev et al., 1998) and at the CP side (Arg^{227} and Asp^{38}; Brown et al., 1994; Riesle et al., 1996; Checover et al., 1997), respectively. We hypothesize that the proton release from the EC cluster and the uptake at the CP cluster involve reorganizations of the participant residues and water molecules. The protonation of amino acid Asp^{85} creates an electric dipole that induces ordering in EC and CP clusters (rise of the probe signal) to conformations able to release (EC side) or uptake and relay protons (CP side) in a second conformational change (decay of the probe signal). These reactions take place far from the neighborhood of the retinal; therefore they do not influence the light absorption signals, i.e., they are silent in the visible but may appear in the infrared. The processes have activation enthalpies different from the known values occurring in the photocycle, and, as ordering phenomena, they have the characteristic negative entropy. These Arrhenius parameters, which should characterize the proton release and relay, are measured with the proton-sensitive probes at the two sides of the pm (Heberle and Dencher, 1992; Heberle et al., 1994; Alexiev et al., 1995). This way, we assign the phenomena characterized with the probe signals to the bR molecule itself and not to surface properties. The probes are protonated as the protein releases the protons from the cluster at the EC side or as they are approaching the CP cluster. We assume that in the latter process the probe is protonated first

because of reorganization of the CP cluster and then transfers the proton to one of the members of the cluster. This hypothesis is an alternative interpretation of the probe data without the difficulties mentioned above and is in agreement with the work of Cao et al. (1995) and Dioumaev et al. (1998), which postulates that protonation of Asp^{85} and proton release are separate phenomena. It may also be related to the fast protonation of Asp^{38} at the CP side (Checover et al., 1997). It goes further, however, by assigning the measured and not yet explained Arrhenius parameters to these processes.

The additional electric signals seemingly reflect the processes in the clusters if the buffers are added in appropriate concentration, salt, and pH. The coincidence of the Arrhenius parameters of the additional electric signals with those measured with the proton-sensitive probe (Table 1) lead us to generalize the hypothesis and argue that the interaction of the clusters and buffers is also responsible for the additional currents.

The surface charge asymmetry of pm, a four-charge difference between the CP and EC sides (Kimura et al., 1984; Nachliel et al., 1996), establishes an uneven microdistribution of the charged buffers in a more or less salt-free solution. At the pH of "good efficiency," i.e., at which the additional charge motion is high, the charge states of buffers are shifted in the direction of deprotonation. Therefore, there are negatively charged Gly-Gly molecules, and singly and doubly positively charged BTP molecules in the solutions. Fig. 9 shows the result of a molecular dynamics calculation (Oroszi et al., unpublished calculations). The method is based on the simulation of the Brownian motion

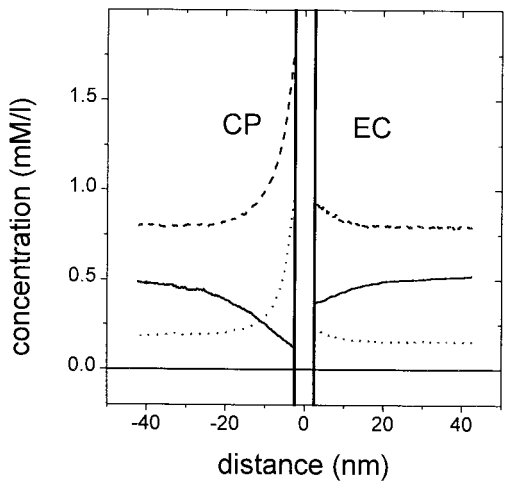

FIGURE 9 Calculated microdistribution of the charged buffers around pm. ——, Singly charged Gly-Gly; – – –, singly charged BTP; ·····, doubly charged BTP. Conditions: one negative charge at the EC surface, five negative charges at the CP surface per bacteriorhodopsin molecule, pH 7.5.

of individual ions in the electric field of the diffuse double layer. Considering the equilibrium conditions calculated with five and one negative charge per bR on the CP and EC sides, respectively, one sees that concentration gradients of the charged buffers build up around the pm. This calculation is planned to solve more complex problems. In equilibrium conditions it yields the same results as the Gouy-Chapman theory. The main difference in the microdistribution of these buffers in addition to the uneven gradients at the two sides is that the positively charged BTP buffer molecules are much closer to the membranes than the negatively charged Gly-Gly buffer molecules and the direction of the concentration gradient is the opposite.

The difference between a buffered and a buffer-free solution, therefore, resides in the uneven, nonhomogeneous microdistribution of the buffers determined by the surface charges of the pm. It is very important to note that buffers are special proton donors and acceptors. As described by Engasser and Horvath (1973) and Gutman and Nachliel (1990), the buffers can efficiently take the protons away from the site or toward the site of reactions. As we mentioned in the Introduction, this property of buffers was used to interpret the speed-up of the response of the dissolved proton-sensitive dyes for proton release (Drachev et al., 1984; Grzesiek and Dencher, 1986; Váró and Lanyi, 1991).

The cases of Gly-Gly and BTP buffer need different treatments. First we deal with the Gly-Gly buffer. As the protons are released from the EC cluster they are picked up by the buffers and guided along the concentration gradient of the negatively charged Gly-Gly molecules shown in Fig. 9. This proton motion is directed perpendicularly to the surface of pm and produces the EC part of the current. At the CP side protons are picked up by the CP cluster from the neutral buffers that become negative and are driven away from the membrane. This process occurs in the microsecond time domain controlled by the reorganization of the cluster as mentioned above. Motion of positive charge at the EC side and negative charge in the opposite direction at the CP side induces the observed positive additional current. Here we accept the results of Alexiev et al. (1995), which indicate the simultaneity of the probe signals at the two sides of the pm. The microdistribution, i.e., the concentration gradient of the charged buffers, is abolished in the presence of salt ions shielding the surface charges. The released protons are accepted or donated by the homogeneously distributed buffers but not guided by their gradients. This way, point 5 above is understandable. In the absence of buffers the protons are released and picked up, but the guiding effect of the buffers, which increases the paths of protons and the charged buffer molecules, is absent.

In the case of the BTP buffer the additional current has microsecond and millisecond components. The Arrhenius parameters of the microsecond component are in the usual range with negative entropy, while the millisecond component has large activation enthalpy and positive activation

entropy. The proton released from the cluster at the EC side diffuses to some distance, and then a singly positively charged BTP molecule picks it up and now, being doubly charged, moves to the membrane generating negative current. This process is responsible for the microsecond components of the additional current. At the CP side protons are picked up preferentially from the doubly charged buffers, and, as singly charged molecules, they move away from the membrane, again generating negative current. This process is responsible for the long-lived component.

The cluster hypothesis, as we call it, explains all seven points summed up above. It offers an alternative explanation for the data obtained with the proton-sensitive probes bound to the surfaces of the pm. It may explain the results obtained with the different buffer groups reported by Liu et al. (1991), just by considering the differences of the gradients around the pm for these buffers. The hypothesis requires further confirmation. The hypothesis in the present form is qualitative; further model calculations are under way in our laboratory (Oroszi et al., unpublished calculations). Some of its consequences may be approached via well-defined experiments like studies of buffer effects on bR mutants modified at either the EC or CP cluster.

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