Photovoltage Kinetics of the Acid-Blue and Acid-Purple Forms of Bacteriorhodopsin: Evidence for No Net Charge Transfer

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ABSTRACT Time-resolved photovoltage measurements were performed with the acid-blue (bR^A_{605}) and acid-purple (bR^A_{565}) forms of bacteriorhodopsin (bR) in the time range from 25 ns to 100 s. The bR^A_{605} and bR^A_{565} pigments were formed by titration with H_2SO_4 in the absence and presence of 150 mM KCl, respectively. Qualitatively the kinetics of the charge displacement in these two states are similar and consist of two fast phases in one direction (100 ns bandwidth limited and ~1 μs) followed by a decay in the opposite direction via one component for bR^A_{605} (4.4 ± 0.6 ms) or two components for bR^A_{565} (33 ± 8 μs and 3.6 ± 0.5 ms). The transient photovoltage signal returns exactly to the initial value after several milliseconds, well before the passive discharge of the electrical measuring system at 2 s. We conclude that no net charge transfer occurs in either bR^A_{605} or bR^A_{565}. The direction of the fast components is opposite that of net proton translocation in bR at pH 7. So, if the charge that moves back and forth is due to a proton, it moves first in the direction of the cytoplasmic side of the membrane (<1 μs) and returns to its initial position via the 4.4 ms (bR^A_{605}) or the 33 μs and 3.6 ms (bR^A_{565}) decay components. The amplitude of the charge motion in both low pH forms is too large to be due to isomerization alone and is comparable to one of the major components in bR at pH 7. From the pH dependence of the photovoltage amplitudes apparent pK_as of 1.6 ± 0.2 and 2.0 ± 0.2 were determined for the transitions from bR_{568} to bR^A_{605} and to bR^A_{565}, respectively.

INTRODUCTION

The absorption maximum of the retinal chromophore of the light-driven proton pump bacteriorhodopsin (bR) shifts from 568 to 605 nm upon acidification of the bulk medium (Oesterhelt and Stoeckenius, 1971; Fischer and Oesterhelt, 1979; Mowery et al., 1979). This so-called "acid-blue" form of bR (bR^A_{605}) is conveniently produced by titration with H_2SO_4. Since the transition depends on the surface pH, which in turn is determined not only by the bulk pH but also by the surface charge of the membrane and the ionic strength of the bulk solution, the apparent pK_a varies with these parameters between 3.5 and 1.4 (Szundi and Stoeckenius, 1987). Titration with HCl leads to the formation of the so-called "acid-purple" form of bR (bR^A_{565}) with an absorption maximum at 565 nm (Fischer and Oesterhelt, 1979; Mowery et al., 1979; Váró and Lanyi, 1989). The pH and anion effects can be conveniently separated by using K_2SO_4 and a constant Cl^- concentration (Renthal et al., 1990; Váró and Lanyi, 1989). In the acid-blue form, aspartate 85, the main component of the counterion to the Schiff base (Otto et al., 1990), is protonated (Metz et al., 1992). In the presence of halide anions at low pH, the purple color is restored, since these small anions can bind directly to the protonated Schiff base forming a surrogate counterion (Fischer and Oesterhelt, 1979; Marti et al., 1991; de Groot et al., 1990). The photocycle of the acid-purple and acid-blue forms of bR lack an M-intermediate with a deprotonated Schiff base (Váró and Lanyi, 1989; Heyn et al., 1989). Because aspartate 85 is also the acceptor of the Schiff base proton at neutral pH (Braiman et al., 1988), no proton transfer can occur between these two groups in the acid forms explaining the absence of an M-intermediate. It is therefore generally assumed that both acid forms are inactive as proton pumps, although pH or pH-dye measurements could not be performed under these conditions.

The availability and properties of mutants of bR have led to renewed interest in the acid forms of bR. For example, when aspartate 85 is replaced by a neutral amino acid, bR is inactive as a proton pump at neutral pH and its absorption maximum is red-shifted (Mogi et al., 1988). Thus these mutants mimic the acid-blue form of bR at neutral pH. When both aspartic acids 85 and 212 in the counterion of the Schiff base are replaced by neutral amino acids, the chromophore can only be regenerated in the presence of halide anions, the halide anions bind directly to the protonated Schiff base, and the λ_{max} value is ~565 nm (Marti et al., 1991, 1992). Thus, these mutants mimic the acid-purple form of bR at neutral pH.

The intramolecular charge movement of the low pH forms of bR have been investigated by voltage (Drachev et al., 1978, 1981) and current (Dér et al., 1989, 1991; Keszthelyi et al., 1990) measurements. The photovoltage work was hampered by an insufficient characterization of the measuring system, whereas the current measurements suffered from the fact that the apparent amplitudes of the exponential processes depend on the respective rates. For photocurrent measurements, the amplitudes of the charge translocation are weighted by the rates, leading to very small amplitudes for the slower components. The photovolt-

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age system used here has the advantage of constant amplitude sensitivity over 7 decades in time from <100 ns to 1 s and of being completely analyzed in terms of the passive system behavior (Holz et al., 1988). The previous photovoltage measurements at low pH showed a rapid unresolved charge movement in a direction opposite that of net proton movement at neutral pH, but did not completely observe the return of the signal and the slow system discharge (Drachev et al., 1978, 1981). It was nevertheless concluded that no net charge transport occurs. In this paper, we reinvestigate the charge translocation in the acid-purple and acid-blue forms of bR with improved methodology and with the following goals. Recently, it was concluded from photocurrent measurements that in the acid-purple form net charge transport of the halide anions Cl^- and Br^- occurs with substantial kinetic isotope effects (Dér et al., 1989, 1991; Keszthelyi et al., 1990). Using photovoltage methods, we show here that the kinetics of the charge displacement in acid-blue and acid-purple bR are quite similar and that in both forms no net charge translocation occurs. A better understanding of the transport properties of the acid forms of bR is furthermore required as a basis for the interpretation of the electrical signals of various "blue" or "acid-purple" mutants. This is the subject of the accompanying paper (Moltke et al., 1995).

MATERIALS AND METHODS

Membrane fragments (purple membranes, PM) containing bR were prepared according to standard procedures and stored in distilled water at −18°C. Before usage the thawed solutions were diluted with our standard buffer mix (3 mM each of HEPES, TRIZMA Base (Sigma Chemical Co., St. Louis, MO) and sodium acetate, and varying amounts of electrolyte and sonicated for 20 s. Absorption spectra were recorded on a Shimadzu UV-VIS spectrophotometer UV-2102PC with suspensions of PM in the same buffer solution as used for the electrical measurements (see below).

Photovoltage measurements

We used a modified version of the experimental setup described earlier (Holz et al., 1988). The techniques used for measuring the charge translocation in bacteriorhodopsin have been reviewed comprehensively (Trissl, 1990). PMs are oriented by adsorption to a lipid-impregnated polyethylene sheet. The lipid coating is a solution of 1.2% (w/v) L-1,2-diphytanoyl-3-phosphatidylcholine (Avanti Polar Lipids, Birmingham, AL) and 0.025% octadecylamine (Fluka (Buchs, Switzerland), puriss) in *n*-decane (Merck, for gas chromatography), giving it a net positive surface charge. The sheet separates two compartments of a DELRIN100 cuvette. After filling the compartments with a standard buffer solution, ~10 nmol bR are added to the compartment on the coated side. The cuvette is kept at a constant temperature of 25°C with a thermostat.

The signals are coupled to the preamplifier via Ag-AgCl electrodes, which are submerged in the same bathing solution but separated from the cuvette by paper-filled, buffer-soaked bridges to shield them from the laser light. A large shunt resistance of 5 GΩ is used to prevent a fast external discharge of the system. A homemade circuit using the operational amplifiers 3554 and 3553 (Burr Brown) amplifies the voltage amplitudes by a factor of 100. The bandwidth of the system is ~10 MHz. The whole setup with battery power supply is housed in a Faraday cage.

The photoreactions are started by a 580 nm dye laser flash (rhodamine 6G) of 25 ns duration reaching the sample via a metal tube to prevent external fields from entering the cage and through a quartz window in the cuvette. The flash is also detected by a fast photodiode to provide the trigger for the data acquisition system.

Digitization of the voltage is performed using two PC boards (T12840 and T512 from IMTEC, Backnang, Germany). These provide two independent time bases (shortest sampling times 25 ns and 200 ns, respectively), amplifiers (minimum full range 256 mV and 125 mV), A/D converters (8- and 12-bit) and on-board memory (64 kb and 1 Mword). The signal is recorded simultaneously in these two trigger-coupled channels. Each data set is reduced to ~470 data points each on a logarithmic time scale, and finally incorporated into one continuous data set by linear regression in the time window common to both channels, allowing for a variation of the amplification factors of a few percent and an internal offset. Averaging 5–20 single shots is sufficient for a signal-to-noise ratio of 200–2000.

Data analysis

The signals are graphically represented on a logarithmic time scale to achieve a concise overview over nine decades from 25 ns to 100 s (Rayfield, 1985). The proper description of the photovoltage in terms of analytic functions is still an open issue. A first-order kinetic analysis of the photoreactions leads to a sum of exponential functions. Up to seven components are required to describe the active pumping processes for a satisfactory fit with no systematic deviations in the time course of the residuals. From the limited reproducibility of these components it has been argued that a model using distributed kinetics is more adequate (Holz et al., 1988). In the present study, both models are applied. We use Gaussian distributions of the rate constants to characterize the signal globally, and simple exponentials if a highly sensitive detection of every possible kinetic component is required. In each case, the residuals are the main criterion for a satisfactory fit to the data. Because of this uncertainty, we cannot interpret the amplitudes in terms of the relative distance the respective charge is moved.

The fit uses a nonlinear, iterative least squares procedure. The quality of the fit is generally evaluated by computing the quotient of the signal amplitude and the mean deviation of the data from the fit curve.

Acidification of the bulk medium

The acidification of the bulk medium necessary to convert bR to the acid-blue or acid-purple state is achieved by adding microliters of highly concentrated H_2SO_4 to both compartments of the cuvette. The pH is measured in the cuvette using a standard glas electrode. Because of the paper filling of the bridges connecting the cuvette to the electrodes, there is at first a pH gradient between the cuvette and the electrode compartments. It takes about 1 day to dissipate by diffusion. Experiments performed with cuvette, bridges and electrode compartments filled with solution at the same low pH have shown no influence of the gradient on the voltage measurements.

RESULTS

The active pumping state at pH 7 and sign conventions

Fig. 1 shows representative photovoltage traces from bR in a solution containing 150 mM KCl. The data at pH 7 are in excellent agreement with previously published results (Holz et al., 1988); the exponential describing the first rise has a negative amplitude A_1 and its time constant is bandwidth limited (Simmeth and Rayfield, 1990). (Because the signal is fitted basically as a sum of exponentials, the preexponen-

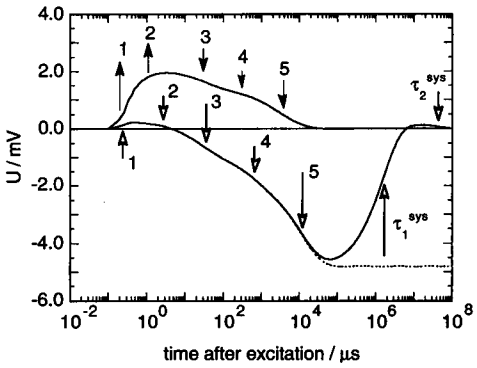

FIGURE 1 Photovoltage of bacteriorhodopsin in standard buffer solution (3 mM each of HEPES, Trizma base, and sodium acetate) and 150 mM KCl at 25°C. The traces are not scaled; the PMs have been adsorbed at pH 7 and titrated to low pH by adding highly concentrated H_2SO_4 in microliter amounts. Arrows indicate the time constants, sign (a downward arrow means positive amplitude of the corresponding exponential; an upward pointing arrow correspondingly means a negative amplitude) and relative amplitudes of the major components of the pumping kinetics. The arrows with hollow tips mark the photovoltage at pH 7; those with filled tips the voltage at pH 0.3. For the values of the fit parameters see Table 1. At pH 7, the system times τ_1^{sys} and τ_2^{sys} at 1.8 s and 50 s, respectively, represent the passive discharge times of the system, which have been deconvoluted in the dotted curve (· · · ·).

tial factor (called amplitude) is positive, if the initial value is higher than the final value for this component, and it is negative if the end value is higher than the starting value, regardless of whether the voltage itself is negative or positive.) It is followed by a small relaxation A_2 with positive amplitude. The next three main components A_3, A_4, and A_5 are fitted with Gaussian distributed rate constants. For processes no. 3 and 5 two exponential functions are needed for each to achieve the same fit quality without distributions, whereas distribution no. 4 can be replaced by a single exponential without significant loss in fit quality. Because of the finite shunt resistance, the system discharges passively after a few seconds with two characteristic exponential decay times τ_1^{sys} and τ_2^{sys}, as described (Holz et al., 1988). The influence of the system can be exactly deconvoluted from the active pumping processes as shown by the dotted line in Fig. 1. Because the final value of the photovoltage is negative (Fig. 1, *dotted line*) and since bR pumps protons to the extracellular side, it can be concluded that the PM adsorbs with the extracellular side to the lipid film.

At pH 7 no significant difference in the photovoltage kinetics is found with the use of the standard buffer solution without any additional salt (data not shown). The only minor change is a slowed rise time of the very first component, $\tau_1 \sim 1$ μs instead of 100 ns, and consequently a barely detectable A_2. In the absence of salt, the resistance of the solution is very high and is now limiting the bandwidth together with the stray capacitance of the electrodes and conducting wires. A similar problem has been discussed earlier (Trissl et al., 1984; Liu and Ebrey, 1988; Trissl, 1990). Using 75 mM K_2SO_4 in the bulk solution also has no significant effect on the kinetics at pH 7 and pH 0.4 (for a comparison of fit parameters cf. Table 1).

The photovoltage at very low pH

Fig. 1 illustrates the dramatic change in the photovoltage upon acidification to pH 0.3 in the presence of 150 mM KCl (bR^A_{565}). There are three major features, which distinguish this signal from the pumping signal at pH 7. The amplitude of the first negative relaxation is greatly increased, but again, the time constant is that of the system rise time (~0.1 μs, −40 ± 6% relative amplitude; this value and the ones following in the text are averaged over five series of titrations. The sum of the absolute values of the relative amplitudes is 100%.) The next process also has a negative amplitude and a time constant of ~1 μs (−10 ± 6%). No relaxation at all is observed in the time range of the passive system discharge, because the voltage returns back to 0 with a time constant of several milliseconds. Similar observations were also made for the blue state bR^A_{605} of the chromophore (produced either in the absence of any salt or by using 75 mM K_2SO_4 in the buffer solution). A comparison of the respective photovoltage kinetics is shown in Fig. 2 *A*. The main effect of the halide anions is on the positive

TABLE 1 Kinetic parameters of the photovoltage of bR*

Conditions	Component no.	A/mV	A/%	τ	σ
150 mM KCl					
pH 7	1	−0.38	−7	180 ns	
	2	0.08	1	4 μs	
	3	1.27	23	34 μs	0.39
	4	0.87	16	730 μs	
	5	2.91	53	12 ms	0.24
pH 0.3	1	−1.39	−34	130 ns	
	2	−0.65	−16	900 ns	
	3	0.66	16	37 μs	0.26
	4	0.08	2	400 μs	
	5	1.32	32	3.7 ms	0.40
75 mM K_2SO_4					
pH 7	1	−0.30	−5	110 ns	
	2	0.22	4	8.8 μs	
	3	1.80	31	34 μs	0.26
	4	0.52	9	350 μs	0.01
	5	3.03	52	13 ms	0.42
	6				
pH 0.4	1	−0.40	−23	130 ns	
	2	−0.45	−26	1.0 μs	
	3	−0.04	−2	2.8 μs	
	4	0.02	1	50 μs	
	5	0.76	43	4.3 ms	0.50
	6	0.08	5	46 ms	

*The two sets of data with 150 mM KCl and 75 mM K_2SO_4, respectively, are each from the same experiment, i.e. the data at pH 7 were recorded immediately after adsorption of the PM and those at low pH after titrating with H_2SO_4. The halfwidth σ of the Gaussian distribution of the rate constants is given in terms of log *k*. The time constants τ refer to the maximum of the distribution. At pH 7, the system discharge has been deconvoluted. Amplitudes of <5% of the total amplitude have large error margins and are not discussed in the present work.

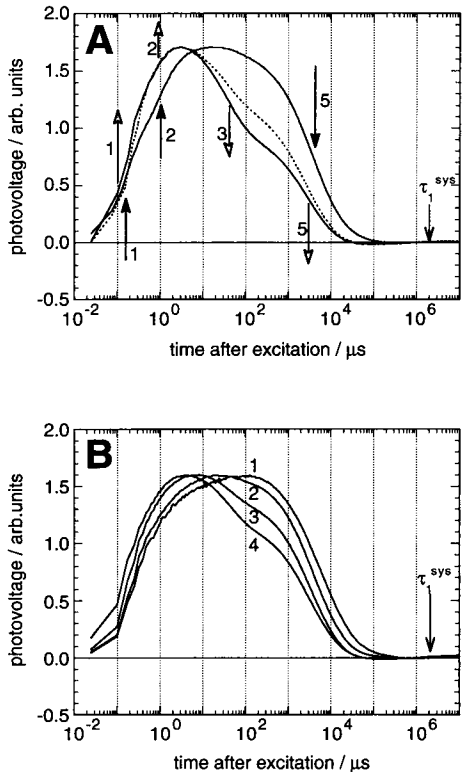

FIGURE 2 (A) Scaled photovoltage traces from wildtype bacteriorhodopsin with varying amounts of Cl^- in the standard buffer solution at 25°C: 75 mM K_2SO_4 at pH 0.4 (—, marked with filled tip arrows), 150 mM KCl at pH 0.3 (· · ·) and 1 M KCl at pH 0.3 (—, marked with hollow tip arrows). The PMs were adsorbed at pH 7, and the bulk solution was then titrated by adding microliters of highly concentrated H_2SO_4. The arrows indicate the time constants, sign (an upward pointing arrow corresponds to a negative amplitude of the exponential), and relative amplitudes of the major fit components. The time constants of both curves measured in the presence of KCl are identical, the amplitudes differ. The components left out in the figure account for <10% of the total signal amplitude. For the fit parameters see Table 1. (B) Scaled voltages from a series of measurements at pH 0.2 in which microliter amounts of a 3 M KCl solution were added to the initially salt-free standard buffer solution with PMs adsorbed at pH 7 and titrated to pH 0.2 with H_2SO_4 (25°C). The KCl concentrations in the cuvette were approximately: (*1*) 0.0 M KCl, (*2*) 0.08 M KCl, (*3*) 0.8 M KCl, and (*4*) 1.6 M KCl. The apparent acceleration of the fast rise of the signal is due to the decrease in electrolyte resistance as KCl is added and the resulting increase in the bandwidth of the system.

phases of the photovoltage. In the absence of these ions in the electrolyte solution, i.e., without any salt or with K_2SO_4, the positive decay of the voltage can be fitted with just one very broad distribution of rate constants centered at 4.4 ± 0.6 ms (45 ± 4%), whereas the decay in the presence of chloride is clearly at least biphasic with two distributions at 33 ± 8 μs and 3.6 ± 0.5 ms. To allow also the comparison of the absolute amplitudes, the fit results for two experiments are summarized in Table 1. The amplitude A_3 depends on the amount of chloride available in the solution, as demonstrated by Fig. 2 *B*. Instead of adding the salt at pH 7, as was done in Fig. 2 *A*, the sample was first titrated in a salt-free solution to pH 0.2 (acid-blue form; *curve 1*), and KCl was then added at this pH in small aliquots. Fig. 2 *B* illustrates that chloride induces the electrical transition from acid-blue (*curve 1*) to acid-purple (*curve 4*) at constant low pH.

Acidification in the absence and presence of chloride

The pH and temperature dependence of the photovoltage kinetics were studied in the absence of salt and in the presence of 150 mM KCl. These conditions were chosen for the following two reasons. First, we have taken the low pH form in the absence of any salt as the blue state reference to exclude any influence of the cations in the solution. Second, even though the conversion to the acid-purple state is not yet complete in 150 mM KCl (see Fig. 2), the photovoltage can be recorded reliably under those conditions, whereas in 1 M salt the signal is distorted by large drifts and the reproducibility of the fit parameters is poor.

To characterize the observed changes in the voltage kinetics more clearly as effects of the acidification, Figs. 3 and 4 show the normalized amplitudes as a function of pH. A data evaluation of this kind is hampered by several difficulties. Changing the pH of the medium leads to a shift in the absorption maximum of the chromophore, which should be compensated by a corresponding shift of the excitation wavelength. This is in practice not possible to perform, because during the titration a mixture of both forms is produced. Also, the quantum yield of the pH 7 and pH 0 states of the chromophore will be different. Third, the degree of orientation due to the adsorption of the PM is certainly far from perfect and might change because of changes in the protonation state of groups on the surface of the membrane. Last, the effects of the acidification will consist in the vanishing of the relaxations dominant at pH 7 and the rise of those arising from the low pH state, i.e., the amplitudes of the pH 7 components will continuously de-

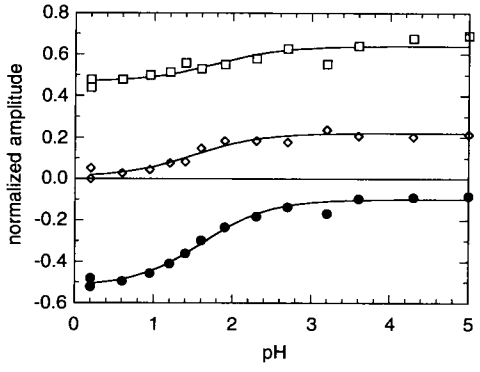

FIGURE 3 The pH dependence of the normalized amplitudes of the fit components A_1 (●), A_3 (◇) and A_5 (□) of the photovoltage from wild-type bR in the absence of salt (under these conditions, the second negative component cannot be resolved because of its small amplitude). The titrations were performed by adding highly concentrated H_2SO_4 to the standard buffer solution at 25°C. The pH dependence is described by the Henderson-Hasselbalch equation; the pK_a values are: pK_1 = 1.6 ± 0.1, pK_3 = 1.5 ± 0.2, and pK_5 = 1.8 ± 0.3.

crease to 0 while those of the pH 0 components will increase to their final value. Because the time constants of the two sets of relaxations are not sufficiently separated, especially when using distributions of rate constants, the fitting procedure does not give reliable values for the respective amplitudes. Instead, one distribution of rate constants will contain contributions from both states, if a pair of charge translocations occurs close together in time. At pH 7 one such distribution will correspond entirely to one of the native pumping processes, and it will end up at pH 0 with an amplitude given only by the respective low pH process, accompanied by some shift on the time constant axis. The change in the amplitude of a distribution reflects the change in the amount of each protein form. Therefore, we have computed the relative amplitudes as the ratio of the amplitude of each component and the sum of the absolute values of all the amplitudes contained in the photovoltage. Finally, we evaluate the pH dependence of the amplitudes corresponding to fit components whose rate constants change by less than a factor of 5 over the whole titration. In this way we achieve only a phenomenological description of the titration behavior. The pH dependence of these relative amplitudes can nevertheless be fitted with the Henderson-Hasselbalch equation for a single protonation reaction. Fig. 3 shows that in the absence of KCl, the apparent pK_a is ~1.6, whereas in 150 mM KCl we obtain a pK_a of 2.0 ± 0.2 (Fig. 4). In 1 M KCl the pK_a increases to 2.8 ± 0.4 (data not shown). Because the acid-blue photovoltage is characterized by a vanishing amplitude A_3 of the 30 μs process, it is interesting to note from Fig. 4 that the amplitude of this component diminishes during the titration in KCl between pH 3 and 2 and rises only subsequently to its final value. This may be evidence for the expected transition sequence $purple_{pH7}$ → blue → acid-purple (cf. below).

Temperature dependence of the time constants at low pH

The photovoltage components that do not depend on the protein, i.e., the risetime-limited first process and the passive system discharge, are known at pH 7 to have no significant temperature dependence. To find any non-active components, a sum of single exponentials is fitted to the signal and the rates are analyzed using the traditional Arrhenius plot. In the presence of 150 mM KCl at pH 1.0 seven exponentials are required for a satisfactory fit (Fig. 5). As expected, in both the absence (data not shown) and the presence of chloride (Fig. 5), the time constants show a linear temperature dependence except for the first component, which changes only very little with temperature. Remarkably, the activation energies found in the absence of salt are consistently lower than in the presence of chloride. With chloride, they are of the same magnitude as those of the active pumping components at pH 7.

Control experiments

The interpretation of the photovoltage in terms of net charge transport and with respect to the direction of the charge movement depends critically on the passive system discharge times and the net orientation of the adsorbed PMs. The amount of adsorbed PM is too low to perform optical or other measurements on the system. Therefore, only indirect evidence can be gathered to prove that these parameters remain essentially unaffected by the experimental procedures.

Two tests were performed to characterize the passive system discharge. A long square pulse (10 s) was applied to the electrodes to charge the polyethylene sheet, and the breakdown of the voltage was measured. Both at pH 7 and at pH 0 the discharging exponential process occurred with a time constant of 1–2 s. According to the published equiva-

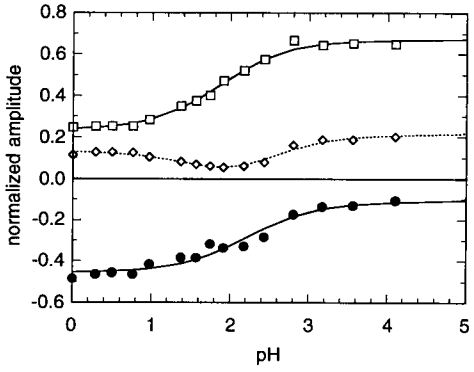

FIGURE 4 The normalized amplitudes of the fit components A_1 (●), A_3 (◇), and A_5 (□) of the photovoltage from wild-type bR in standard buffer solution with 150 mM KCl at 25°C. The solution was acidified by adding H_2SO_4. The pH dependence of the amplitudes is described by the Henderson-Hasselbalch equation. The pK_a values are: pK_1 = 2.2 ± 0.2, and pK_5 = 1.9 ± 0.1.

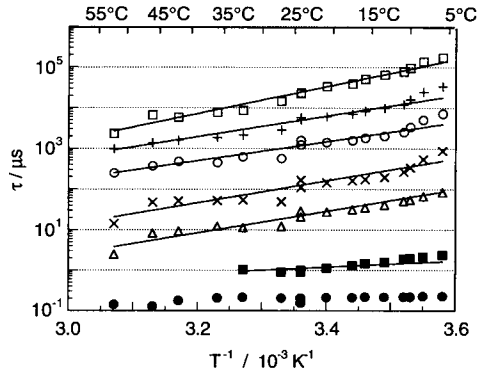

FIGURE 5 Arrhenius plot of the time constants of a seven-exponential fit of the photovoltage at pH 1.0 in standard buffer solution with 150 mM KCl. While the bandwidth limited time constant τ_1 is virtually temperature independent, the activation energies for the other components are: $E_a(\tau_2)$ = 14 kJ/mol, $E_a(\tau_3)$ = 51 kJ/mol, $E_a(\tau_4)$ = 52 kJ/mol, $E_a(\tau_5)$ = 46 kJ/mol, $E_a(\tau_6)$ = 49 kJ/mol, $E_a(\tau_7)$ = 64 kJ/mol.

lent circuit analysis (Holz et al., 1988), this is exactly the value expected, because we used a shunt resistance of 5 GΩ and measured a sheet capacitance of ~300 pF. The conductivity of the sheet did not increase as a consequence of the acidification.

Both voltage and current amplitudes depend on the amount of charge detected by the respective method and are directly related to each other, the voltage being proportional to the integral over the current. Voltages can only be recorded up to the passive discharge, whereas currents are independent of this limitation. In the case of a very fast passive discharge function at pH 0, the voltage measurement would detect a smaller amount of charge moved than the current measurement. The best comparison of the two methods is by direct numerical integration of the current to give the corresponding voltage. This was not successful in our case because of the large artifact due to the system short circuit, which distorts the current signal at low pH in the microsecond range. Only after this short circuit true photocurrents are measured (Trissl et al., 1984). However, a fit of the current trace gives at least no evidence for slower processes than those detected in the photovoltage, and the voltage and integrated current amplitudes are in reasonable agreement for times $>10\mu s$.

The degree of orientation is the least accessible of all parameters in a voltage measurement. Two observations should be pointed out in this context. First, the effects produced by acidification are completely reversible; second, the absolute amplitudes at pH 7 after titration to low pH and back again show no systematic variation. In some cases they are somewhat smaller, whereas in others they are larger than at the beginning. If there were a reorientation taking place in the acidic medium, a systematic effect would be expected.

DISCUSSION

The main conclusion from our experiments is that no net charge transport occurs in either the acid-blue or acid-purple state of bR. This conclusion was based on the observation that the transient photovoltage returns to the baseline in a few ms, well before the passive system discharge, which takes place in several seconds at pH 7. A number of arguments and control experiments support our view that these ms components are due to active intramolecular charge transfer and do not represent an accelerated system discharge. The major slow electrical relaxation times of 3.7 and 4.3 ms for the acid-purple (bR^A_{565}) and acid-blue forms (bR^A_{605}) respectively, (Fig. 2) correlate well with the times for the last steps of the corresponding photocycles (Váró and Lanyi, 1989). This argues against these times being passive discharge times. Moreover their activation energies are large (Fig. 5) and comparable to those observed at neutral pH for active proton transfer steps. No appreciable temperature dependence is expected for an inactive system time. At pH 0 a square voltage pulse applied to the polyethylene support decayed with a time of 1–2 s, which argues against a fast discharge within a few milliseconds over the shunt resistance. Moreover, integration of the current signal at pH 0.45 agreed quite well with the measured voltage signal in the time domain after the system short circuit under voltage clamp conditions, suggesting that every charge translocation is properly accounted for.

Concerning the sign and direction of the charge movement, it may be argued that by lowering the pH the adsorbed purple membranes change their orientation and adsorb at pH 0 preferentially with the opposite side of the membrane as compared with the situation at pH 7. The reversibility of the effect of acidification on the photovoltage makes this very unlikely. Moreover, mutants such as D85N or D85A, which at pH 6 are spectrally blue and inactive as a proton pump, show the same sign and kinetics for the photovoltage as reported here for the acid-blue form (Otto et al., 1990; Moltke et al., 1995). This observation is strong evidence against a reversal of orientation of wild-type bR at low pH. The close similarity between the color and photovoltage traces for D85N at pH 6 and wild-type at pH 0 in sulfate is to be expected, since in both systems the charge of aspartate 85 has been removed (by mutation or protonation, respectively). It is very unlikely that the photovoltage for wild-type at pH 0 is affected by a fast system discharge, because the photovoltage experiments with D85N were performed at pH 6, at which the system discharge is normal.

There are two major differences between the electrical signals from bR^A_{605} and bR^A_{565}, one in the kinetics and the other in the absolute amplitudes. The kinetic difference due to the chloride, as is shown by the experiment from Fig. 2 *B*, lies in the appearance of the 33 μs process and can serve as an indicator for each of the two chromophore states. The other difference concerns the absolute magnitude of the low pH signal as compared with the pH 7 signal, as is seen from the values given in Table 1. Whereas in 150 mM KCl the sum of the positive amplitudes is 5.13 mV at pH 7 and the sum of the negative amplitudes at pH 0.3 is −2.04 mV, i.e., 40% of the pumping signal, the corresponding values in 75 mM K_2SO_4 are 5.57 mV for the positive amplitudes at pH 7, and −0.99 mV for the negative amplitudes at pH 0.4, i.e., 18%. Thus, if we take the amplitudes to be proportional to the distances the charge is moved, and if we assume the charge to be a proton, at pH 7 as well as at low pH, the translocation distance is smaller in the acid-blue form than in the acid-purple state. This comparison should not be carried too far, because the absolute amplitudes at pH 7 are not exactly the same upon titrating back from the low pH regime.

From optical absorption measurements the apparent pK_a values of the purple-to-blue transition and the blue-to-acid-purple transition are known to be 3.2 and <2, respectively, while this sequence seems to be reversed here (Figs. 3 and 4). To assess the pK_a values given by our Henderson-Hasselbalch analysis, two points have to be considered. First, the formation of the blue state bR^A_{605} is due to the protonation of aspartate 85 (Metz et al., 1992), presumably from the extracellular side of the membrane. This is indeed

a genuine protonation reaction, whereas the acid purple state bR^{A}_{565} is generated by the binding of Cl^- near the protonated Schiff base, serving as a surrogate counterion. This transition to the acid-purple state is only indirectly pH dependent, as it requires the protonation of aspartate 85 before the binding of the chloride. It was shown that the chloride binding constant increases with decreasing pH (Renthal et al., 1990). The titration to bR^{A}_{565} under conditions of constant chloride concentration as in our experiments is in fact a titration of the purple-to-blue transition with a subsequent conversion of the blue state to the acid purple one. The simple analysis performed here should give mainly the pK_a of the first process, namely the purple-to-blue transition. Second, the system of PM adsorbed to the lipid-impregnated polyethylene sheet differs from that of PMs immobilized in an aqueous gel, as used for the optical titration experiments and the photocurrent studies. The lipid impregnation is in contact with the extracellular side of the PM, i.e., exactly that side from which aspartic 85 receives its proton. The lipid film has a positive surface charge, because the lecithin is zwitterionic and the octadecylamine carries a positive charge. Thus, the pH at the surface of the film will be higher than in the bulk phase. The pH at the extracellular side of the PM, which is in close contact with the positively charged support, will therefore also be higher than at the surface of isolated PMs in aqueous suspension or a gel, which are used in the optical titrations. Consequently, the apparent pK_a of the purple-to-acid-blue transition will be lower in our system of adsorbed membranes than the one determined optically. This explains why, in the absence of salt, we find electrically a pK_a of 1.6, somewhat higher than the pK_a of 1.4 observed optically after removal of the negative surface charge of the PM (Szundi and Stoeckenius, 1987, 1988), but much lower than the pK_a values of 3–4 observed in gels or suspensions of isolated PMs. The positive charges of the film can be shielded by soluble anions. Because in the presence of chloride, the electrically measured pK_a still corresponds to the purple-to-blue transition, as argued above, we expect the apparent pK_a to increase as the ionic strength of the solution is increased. The pK_a indeed increases from 2.0 in 150 mM KCl to 2.8 in 1 M KCl. The anions of the acid used for the titration will have no effect, because they only reach the 100 mM range at a pH <1, clearly below the transition pK_a values.

In agreement with Dér et al. (1991) and Drachev et al. (1978), we find that no net charge transport occurs in the acid-blue state of bR. Dér et al. (1991) and Keszthelyi et al. (1990) did observe, however, net charge translocation for the acid-purple state. Moreover, they showed that with Br^- as the anion, the millisecond electrical components in their photocurrent signals were slower than with Cl^-. Together with their finding of net charge transport, this led them to conclude that in the acid-purple form of bR halide anions are transported in the same direction as in the light-driven chloride pump halorhodopsin (Dér et al., 1991). In our photovoltage measurements we observed no net charge motion and no halide anion isotope effects (data not shown). We therefore conclude that bR does not transport chloride in the acid-purple form. In the following we discuss the possible sources of this discrepancy.

As explained in the Introduction, photovoltage measurements are the method of choice with slow charge motion. For photocurrent measurements, the errors in the amplitudes and times are large for the millisecond components. The amplitudes of the charge motions observed by Dér et al. (1991) in the acid-purple form in HCl at pH 0.5 are listed in their Table 2. The single fast backward charge motion has an amplitude of -28.1 (arbitrary units). The sum of the three forward amplitudes is 29.3. The absolute values of these numbers differ only by 4%. Considering the errors, these two charge movements are within experimental error equal and cancel. The conclusion of no net charge movement is thus fully justified from these data, in complete agreement with our results. If the small difference between 29.3 and 28.1 were to represent the charge motion of Cl^- across the membrane, there is a very serious problem in explaining the meaning of the $\sim$25 times larger charge movement in the forward direction and back again. The kinetic isotope effects observed by Dér et al. (1991) and Keszthelyi et al. (1990) occur only in the two slowest components of the photocurrent, which are most difficult to measure accurately. When Cl^- is replaced by Br^-, these times increase from 0.92 and 11 ms to 3.7 and 17 ms (Table 2 of Dér et al., 1991). Recently, it was shown that Cl^- is more effective than Br^- in converting the acid-blue to the acid-purple form (Renthal et al., 1990). Our data of Fig. 2 show that the return of the electrical signal to the baseline is slower in the acid-blue than in the acid-purple form (4.3 ms and 46 ms vs. 33 μs and 3.6 ms; Table 1). Therefore, the isotope effects detected by Dér and colleagues may be simply explained by noting that with Cl^- they were observing the acid-purple state (fast millisecond kinetics), whereas with Br^- their sample was still partially in the acid-blue form (slower ms kinetics). Moreover, the microsecond component characteristic for the acid-purple form (33 μs in our measurements, 53 μs in Dér et al. (1991) and 34 μs in Keszthelyi et al. (1990)), made up 28% of the decay amplitude in HCl, but only 10% in HBr (Table 2 of Dér et al., 1991), again suggesting a reduced amount of acid-purple in the HBr sample. It is thus likely that these isotope effects are only apparent. We note that there is good agreement between the time constants obtained with the two photoelectrical methods. The time constants of 34 μs and 1.95 ms from the photocurrent measurements in HCl (Keszthelyi et al., 1990) agree well, e.g., with the values of 33 μs and 3.6 ms obtained from the photovoltage measurements in 150 mM KCl.

In both acid forms, a rapid unresolved charge motion occurs in the direction opposite to that of the net charge translocation at pH 7. This component is probably associated with the K-like intermediate in the corresponding photocycles (Váró and Lanyi, 1989). The acid-purple photocycle has an O-like intermediate, which forms in <1 μs and decays over several decades of time with characteristic

times of 10 μs and 1 ms (Váró and Lanyi, 1989). These photocycle times may be correlated with the two phases in the electrical relaxation of the acid-purple form, which we observe around 33 μs and 3.6 ms. The acid-blue photocycle on the other hand has an L-like intermediate, which returns in 5 ms to the initial state (Váró and Lanyi, 1989) and which is probably associated with the single 4.3 ms electrical component observed here.

The amplitude of the initial rapid charge displacement at low pH is much larger than that of A_1 observed at pH 7 and is too large to be due only to the charge separation associated with the photoisomerization. Because it occurs also in salt-free medium at pH 0, there is most likely a contribution by protons. Since the Schiff base does not deprotonate, the source of the protons is unclear. Based on recent observations with the D212N mutant (Cao et al., 1993), one possibility is that as a consequence of the isomerization-induced movement of the SB proton, the pK_a of an internal group drops and a proton is moved in the direction of the cytoplasmic side of the membrane. The initial state is restored by reisomerization and the return of the proton to the initial site. In the accompanying paper, the photoelectrical properties of various mutants are investigated at low pH to gain further insight into the nature of this transient charge transfer (Moltke et al., 1995).

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