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Intramolecular Charge Transfer in the Bacteriorhodopsin Mutants Asp85→Asn and Asp212→Asn: Effects of pH and Anions

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ABSTRACT The photovoltage kinetics of the bacteriorhodopsin mutants Asp212→Asn and Asp85→Asn after excitation at 580 nm have been investigated in the pH range from 0 to 11. With the mutant Asp85→Asn (D85N) at pH 7 no net charge translocation is observed and the signal is the same, both in the presence of Cl^- (150 mM) and in its absence (75 mM SO_4^{2-}). Under both conditions the color of the pigment is blue (λ_{max} = 615 nm). The time course of the photovoltage kinetics is similar to that of the acid-blue form of wild-type, except that an additional transient charge motion occurs with time constants of 60 μs and 1.3 ms, indicating the transient deprotonation and reprotonation of an unknown group to and from the extracellular side of the membrane. It is suggested that this is the group XH, which is responsible for proton release in wild-type. At pH 1, the photovoltage signal of D85N changes upon the addition of Cl^- from that characteristic for the acid-blue state of wild-type to that characteristic for the acid-purple state. Therefore, the protonation of the group at position at 85 is necessary, but not sufficient for the chloride-binding. At pH 11, well above the pK_a of the Schiff base, there is a mixture of "M-like" and "N-like" states. Net proton transport in the same direction as in wild-type is restored in D85N from this N-like state. With the mutant Asp212→Asn (D212N), time-resolved photovoltage measurements show that in the absence of halide ions the signal is similar to that of the acid-blue form of wild-type and that no net charge translocation occurs in the entire pH range from 0 to 11. Upon addition of Cl^- in the pH range from 3.8 to 7.2 the color of the pigment returns to purple and the photovoltage experiments indicate that net proton pumping is restored. However, this Cl^--induced activation of net charge-transport in D212N is only partial. Outside this pH range, no net charge transport is observed even in the presence of chloride, and the photovoltage shows the same chloride-dependent features as those accompanying the acid-blue to acid-purple transition of the wild-type.

INTRODUCTION

Bacteriorhodopsin (bR) is a light-driven proton pump that converts part of the electromagnetic energy absorbed by its retinal chromophore into an electrochemical proton potential across the membrane. The source of the protons is the protonated Schiff base (SB) linkage by which the chromophore is covalently bound to the protein. The SB is buried in the hydrophobic interior of the protein, approximately halfway across the membrane (Hauss et al., 1990). The negatively charged carboxyl groups of aspartate 85 and aspartate 212 are <5 Å away from the protonated SB (Henderson et al., 1990). Together with the positively charged guanidinium group of arginine 82 these residues maintain charge neutrality and form a complex counterion (Otto et al., 1990; de Groot et al., 1990; Marti et al., 1991, 1992).

Absorption of light by bR leads to isomerization of the chromophore and a transient drop of the pK of the SB. This results in the deprotonation of the SB and proton transfer to Asp 85 on the microsecond timescale in the transition from the L to the M intermediate (Braiman et al., 1988). Roughly concomitantly a proton is released on the extracellular side of the membrane (Alexiev et al., 1995). However, Asp 85 remains protonated until the very last step of the photocycle, the millisecond transition from the O intermediate to bR (Bousché et al., 1992), so that the proton that is released on the microsecond timescale must originate from some other group. This currently unknown group is commonly denoted by XH. The deprotonated X is presumably reprotonated from Asp 85 in the O-to-bR transition. In the normal proton pumping cycle Asp 85 thus serves as a key acceptor/donor group in the proton release pathway. Asp 212 on the other hand remains deprotonated throughout the entire photocycle and, besides forming part of the counterion, its role remains obscure.

Time-resolved photoelectric experiments with wild-type bR indicated two major charge movements occurring on the micro- and millisecond timescales that correspond to proton transfer reactions 1) from the SB to the extracellular surface via Asp 85 and XH and 2) from the cytoplasmic surface to the SB, respectively (for a review see Trissl, 1990). Since Asp 85 and Asp 212 are important residues involved in proton transport and form part of the SB counterion, it is of particular interest to study the charge movement in the mutants D85N and D212N in which the negatively charged carboxyl groups have been removed.

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The light-induced intramolecular charge translocation in bR shows characteristic changes in its voltage kinetics when the bulk solution is acidified in the absence of chloride (Drachev et al., 1978, 1981; Moltke and Heyn, 1995). These changes are concomitant with a change in the absorption maximum of the chromophore retinal from 568 nm at pH 7 to 605 nm at pH 3 or lower. We report here the results of photovoltage experiments with the mutants D85N and D212N of bR, which show a red-shifted absorption maximum of the chromophore even at neutral or higher pH. These studies were performed to clarify the unknown nature and origin of the charge motions in the acid-blue form and to elucidate the role of Asp 85 and Asp 212 for the proton transport in wild-type.

The low pH transition of bR from a purple to a blue pigment is generally attributed to the protonation of Asp85 (Otto et al., 1990; Marti et al., 1992). This accounts for the direction of the color shift, since Asp85 is believed to be the major component of the counterion to the protonated SB (Otto et al., 1990; Marti et al., 1991, 1992; Metz et al., 1992). Asp85 is also the acceptor of the SB proton in the L-to-M transition (Braiman et al., 1988; Metz et al., 1992). Thus, it is clear that with the acceptor site occupied in the blue state, the photocycle does not have an M intermediate, and no net charge transport occurs (Moltke and Heyn, 1995). Consistent with these interpretations, the pigment is blue at neutral pH in the mutants D85N and D85A, which contain neutral side chains at position 85 and it is completely inactive (Mogi et al., 1988). Photovoltage measurements confirmed that no net charge transport occurs in D85N (Otto et al., 1990). However, in both the low pH acid-blue form of wild-type and the mutant D85N at neutral pH transient charge displacements are observed, the nature of which are not currently understood. The fact that the pK_a of the SB is lowered from >13 to 7 in D85N and D85A (Otto et al., 1990; Marti et al., 1992; Turner et al., 1993) suggested that Asp85 is the major component of the counter ion to the SB. Charge changes in the residues R82 and D212, which are also believed to be part of the complex counterion (de Groot et al., 1990), have a much smaller effect on the pK_a of the SB and the color shift (Otto et al., 1990; Marti et al., 1991, 1992). The mutant D212N is still partially active (Mogi et al., 1988). In the presence of chloride, this mutant has a purple color at pH 5 and a red-shifted absorption maximum at high pH (Needleman et al., 1991). This inverted purple-to-blue transition at alkaline pH cannot be explained by the protonation of Asp85. The red shift of only ~15 nm is much smaller than that of D85N or the acid-blue form of wild-type. Time-resolved photovoltage measurements showed that in the high pH blue form of D212N no net charge transport occurs (Moltke et al., 1992).

In wild-type anion specific effects are observed at low pH. Whereas at pH 0 the pigment is blue in the presence of sulfate, it is purple in the presence of chloride. There is no net charge transport with either anion present, but the transient charge motion is distinctive for the two forms (Moltke and Heyn, 1995). There is evidence from Fourier transform infrared (FTIR) studies that Asp212 is protonated in the acid-purple form of wild-type (Renthal et al., 1990).

The photovoltage kinetics of the two mutants D85N and D212N were investigated in the present study under current clamp conditions over the pH range from 0 to 11. We were interested in several aspects that led to the following questions. First, is there any correlation between the color of the chromophore and the photovoltage kinetics? Second, do these mutants still show characteristic anion effects? Third, what is the nature of the transient charge motions in the inactive forms of these mutants? Fourth, can net charge transport be restored in the purple forms of these mutants?

MATERIALS AND METHODS

Sample preparation and titration

The expression and purification of bR mutants from a recombinant *Halobacterium salinarium* strain has been described earlier (Krebs et al., 1993). Suspensions of purified membrane fragments containing the mutant proteins were kept at 4°C. Before the experiment they were diluted 1:1 with the standard buffer mix used for all the experiments described here (3 mM each of HEPES, TRIZMA base (Sigma Chemical Co., St. Louis, MO) and sodium acetate) at pH7. The buffer contains either 150 mM KCl or 75 mM K_2SO_4. The samples were then sonicated for about 20 s. Absorption spectra were recorded on a Shimadzu UV-Vis spectrophotometer using the same buffer solution. The samples were titrated with either H_2SO_4 or KOH to keep the halide concentration, if any, constant throughout the series.

Photovoltage measurements

The photovoltage technique used for the present report was described earlier (Moltke and Heyn, 1995; Holz et al., 1988). The general features of time-resolved photoelectrical methods have been reviewed comprehensively (Trissl, 1990). The membrane fragments adsorb spontaneously to a lipid-impregnated polyethylene support if added to the buffer solution in one compartment of the cuvette. This results in a net orientation of the membranes. The compartment on the other side of the sheet is connected to the ground potential. The charge translocation inside the protein charges the supporting sheet leading to a potential change, which is measured via two Ag/AgCl electrodes. All the experiments are performed in a Faraday cage and the cuvette is kept at a constant temperature of 25°C.

After excitation of the sample with a flash at 580 nm from a dye laser, the induced photovoltage is recorded from 25 ns until >100s. The two electrodes are connected via a large shunt resistance of 5 GΩ. The passive system discharge is characterized by the two time constants τ_1^{sys} and τ_2^{sys} (Holz et al., 1988). τ_1^{sys} corresponds to the discharge of the supporting-sheet capacitor over the shunt resistance. With a support capacitance of 300 pF, τ_1^{sys} is ~1.5 s. τ_2^{sys} is due to the passive proton flow around and through the purple membrane (tight seal with support) and has values between 10 and 100 s. Both system discharge times are well understood in terms of the equivalent circuit analysis (Holz et al., 1988). The lower limit of the time resolution in our experiments is thus 1.5 s. Charge transport processes occurring after this time can no longer be measured. On the other hand, if the characteristic system discharge is observed in the experiment, it must be concluded that up to this time a net charge transfer has occurred in the protein. If there is evidence that processes slower than the passive system discharge occur (e.g., from spectroscopic measurements), the charge translocations corresponding to these processes cannot be observed. If the system discharge is not observed, i.e., the signal has returned faster to its initial value, this is unequivocal evidence for an active discharge due to the protein and signifies that no net charge transport occurs. A more detailed account of the technique can be found in the accompanying paper (Moltke and Heyn, 1995).

Data analysis

The photovoltage kinetics are fitted with a sum of exponential functions with or without Gaussian distributed rate constants (Moltke and Heyn, 1995; Holz et al., 1988). Sign conventions are the following. First, since the ground potential is fixed on the side of the supporting sheet without adsorbed membranes and the membranes presumably adsorb with their extracellular surface, a net transport of positive charge toward the extracellular side produces a negative potential of the signal electrode. (The orientation of the adsorbed membranes is evident in the case of wild-type bR, because the direction of net proton translocation is known. Since the membrane fragments obtained for the mutants are from the same organism, and because the mutation does not change the surface charge, we assume that the orientation of the mutants is the same as for wild-type. The same assumption was made for the D85N and D85T mutants adsorbed to black lipid membranes (Tittor et al., 1994).) Therefore, the end value of the voltage after deconvolution of the system discharge functions is negative. Because the signal is fitted basically as a sum of exponentials, the preexponential factor (called amplitude) is positive if the initial value is larger than the final value of this component, and it is negative if the final value is smaller than the initial value, regardless of whether the voltage itself is negative or positive. Wherever the components of the photovoltage trace are marked by arrows in a figure, an upward pointing arrow corresponds to a negative amplitude, and a downward pointing arrow to a positive amplitude.

RESULTS

Asp85→Asn

At neutral pH the mutant D85N is in a blue state (λ_{max} = 615 nm). Fig. 1 shows that the photovoltage at pH 6.7 has the same overall features as that of the low pH blue form of wild-type, i.e., a rapid negative initial rise, a return to the baseline in ~20 ms and no net charge translocation. There are, however, two striking differences compared with the wild-type photovoltage kinetics at low pH (see Figs. 1 and 2 of the accompanying paper (Moltke and Heyn, 1995)). The major difference is the pair of additional charge movements at 60 μs (distributed kinetics) and 1.3 ms (single exponential). These are indicated by arrows in Fig. 2. A further minor difference, which is highly reproducible, is the relaxation at ~1 s with a very small amplitude (Figs. 1 and 2) and which hints at a passive discharge of a small amount of charge through the shunt resistance. As the comparison between the electrolytes KCl and K_2SO_4 of Fig. 2 shows, the photovoltage at pH 7 does not depend on the nature of the anion in the buffer solution. The dotted line in Fig. 2 represents a simulated signal that models the initial fast negative (0.2 μs and 1.2 μs) and the slowest positive (16 ms) components in the D85N signal with only two amplitudes of equal and opposite direction at 0.35 μs and 26 ms. This simulation shows that the pair of relaxations marked by arrows in Fig. 2 are superimposed on a photovoltage signal that is characteristic for the acid-blue state of wild-type (Moltke and Heyn, 1995). The only difference is that the return to the baseline is somewhat slower in D85N at pH 7 (16 ms) than for the acid-blue form at pH 0.3 (4 ms). A fit of the D85N photovoltage kinetics at pH 7 reveals that the two components at 60 μs and 1.3 ms have almost equal amplitudes. They appear in the signal if the pH is raised above 6.5 and remain unchanged relative to the other components up to pH 8.5, where the amplitude of the voltage signal is almost 0. Raising the pH further gives rise to very different voltage kinetics (cf. Fig. 1 and below).

At pH 1, these two features are absent (Fig. 3). Instead, the voltage kinetics are identical to those of the acid-blue form of wild-type, if sulfate is used as an electrolyte, or to those of the acid-purple form of wild-type, if chloride is the anion present (Moltke and Heyn, 1995). The fast rise of the signal consists of two exponential processes. The first one is not resolved in time and the second has a time constant of ~1 μs. This is followed by one relaxation to the baseline

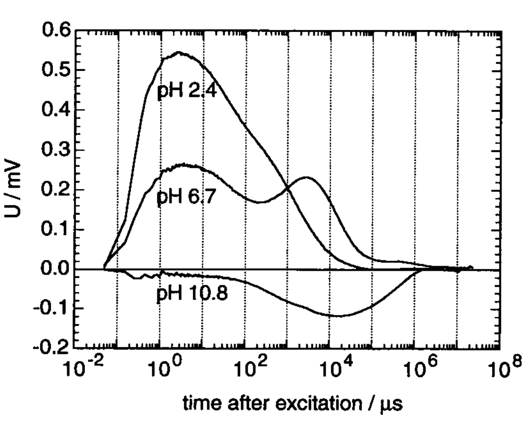

FIGURE 1 Time-resolved photovoltage signal of the bR mutant D85N at three different pH values in standard buffer with 150 mM KCl at 25°C. The pH was adjusted with H_2SO_4 and KOH. The horizontal coordinate is the time after the flash on a logarithmic scale.

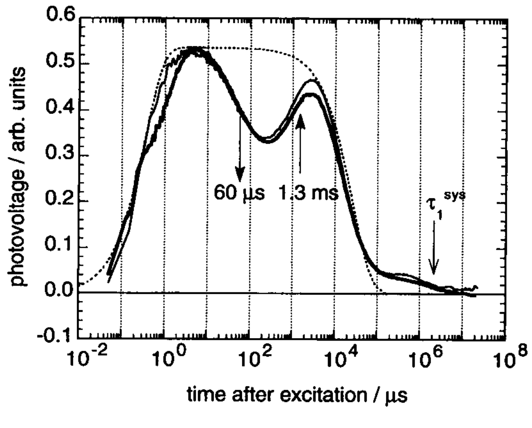

FIGURE 2 Comparison of the photovoltages for D85N at pH 7 in standard buffer (25°C) with 150 mM KCl (*thin line*) and 75 mM K_2SO_4 (*thick line*), respectively. Two exponential processes are marked with their time constants; an upward pointing arrow corresponds to a negative amplitude and vice versa. The arrow marked τ_1^{sys} represents the time constant of the passive system discharge. The dotted curve is a simulation with two exponential functions having amplitudes of equal absolute value but opposite sign and time constants of 0.35 μs and 26 ms, respectively.

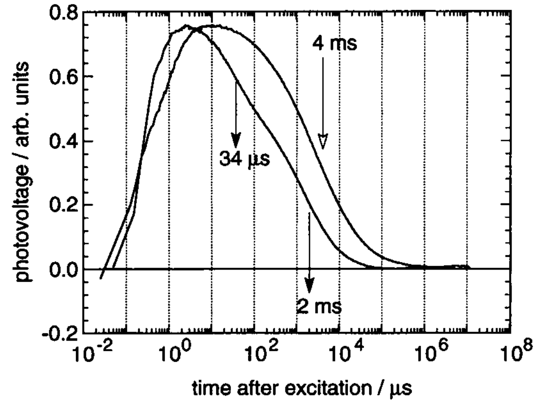

FIGURE 3 Comparison of the photovoltages from D85N at pH 1 in standard buffer (25°C) with 150 mM KCl (*arrows with filled tips* mark the main components with positive amplitude) and 75 mM K_2SO_4 (*hollow tip arrow*), respectively. The pH was adjusted with H_2SO_4. The positive decay of the voltage can be fitted with just one Gaussian distribution of time constants in the case of K_2SO_4, while two distributions are needed for the chloride-dependent signal.

with a broad distribution of time constants centered at 4 ms in the absence of chloride, whereas two distributions at 34 µs and 2 ms are required in the presence of chloride.

Above pH 10, the photovoltage is changed completely (Fig. 1). Not only has the voltage the opposite sign, but two relaxations are observed with time constants corresponding to the passive system discharge. The absolute amplitude at this high pH is diminishing in the course of the experiment, which may be due to bleaching of the pigment. Therefore, the signal-to-noise ratio is poor and the unavoidable drift in the voltage distorts the signal on the time scale of 100 ms and longer. Consequently, the resolution of the slow components is not very good. Nevertheless, we can be sure that net charge transport occurs up to ~100 ms. Assuming that the signals are due to protons, the direction of transport is the same as in wild-type. In KCl, the main components have time constants of $\tau_1 = 0.1$ µs (bandwidth limited), $\tau_2 = 480$ µs, and $\tau_3 = 10$ ms. The amplitude ratio A_2:A_3 lies between 2:1 and 3:1.

Asp212→Asn

Fig. 4 *A* shows representative photovoltage traces for the mutant D212N at several pH values in the presence of chloride. At pH values <3 and >7.5, we observe no relaxation in the time domain of the passive system discharge (marked as τ_1^{sys} and τ_2^{sys} in Fig. 4). The voltage returns to its initial value, i.e., the baseline, before the onset of the passive discharge, indicating that no net charge transfer is occurring. At pH 5.1 on the other hand (Fig. 4 *B*), the system discharge is clearly observed and its time constants are in excellent agreement with those expected theoretically (see Photovoltage Measurements, above). The positions of the five relaxation times required in the multiexponential fit with Gaussian distributed rate constants are indicated by

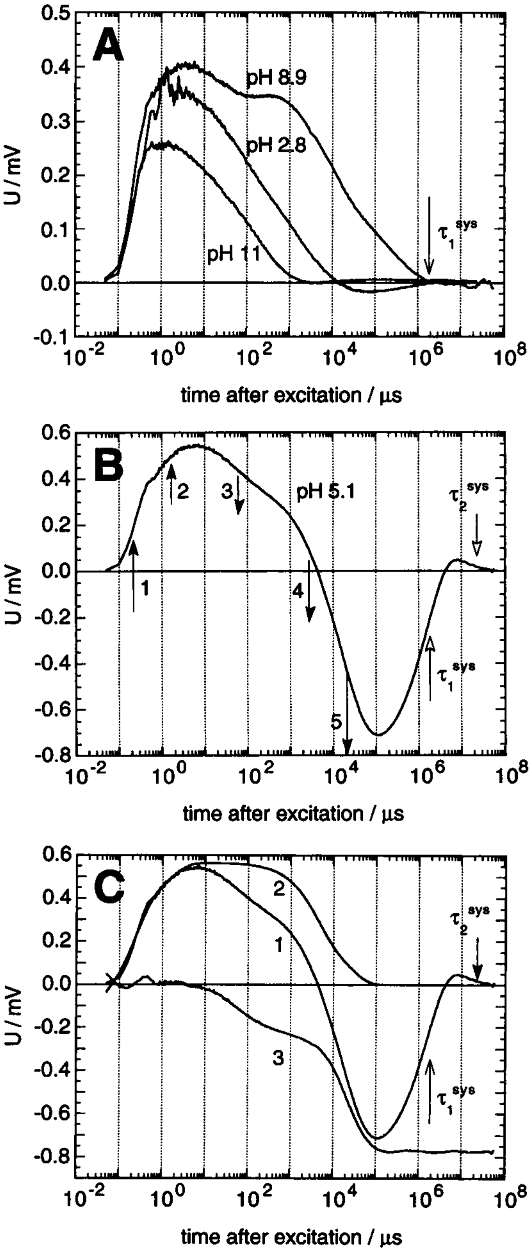

FIGURE 4 (*A*) Photovoltage traces of the bacteriorhodopsin mutant D212N at three different pH values in standard buffer with 150 mM KCl at 25°C. The pH was adjusted with H_2SO_4 and KOH. The arrow labeled τ_1^{sys} marks the time constant of the passive system discharge. (*B*) The photovoltage of D212N at pH 5.1. The individual components are marked with arrows, which point upward if the corresponding amplitude has a negative sign and downward if the sign is positive. The fitted time constants with amplitudes in parentheses are the following: $\tau_1 = 0.2$ µs (bandwidth limited) (−0.52 mV), $\tau_2 = 1.6$ µs (−0.20 mV), $\tau_3 = 68$ µs (0.21 mV), $\tau_4 = 3.8$ ms (0.34 mV), and $\tau_5 = 23$ ms (0.79 mV) plus the two system discharge processes τ_1^{sys} and τ_2^{sys}. (*C*) The photovoltage of D212N at pH 5.1 (*1*) and its decomposition into a blue component (*2*) and a signal corresponding to active pumping (*3*). Curve 1 is the measured data; curve 2 is generated by taking the first two negative components of (1), all of the fourth process, and part (0.23 mV) of the amplitude A_5 so as to return the voltage to the baseline. Curve 3 is the difference between curves 1 and 2; in addition the system discharge τ_1^{sys} and τ_2^{sys} has been subtracted out. Curve 3 clearly shows the net transport and is in excellent agreement with the wild-type active pumping signal (Moltke and Heyn, 1995).

arrows in Fig. 4 *B*. The lengths of the arrows are proportional to the corresponding amplitudes A_1–A_5, which are listed in the figure legend. Compared with the photovoltage of wild-type at pH 7 (Moltke and Heyn, 1995), which is due to the transfer of a proton across the membrane, the fast rise A_1 of the signal has a much larger amplitude and there is a second component A_2 with negative amplitude (upward pointing arrow no. 2) at ~1 μs. The charge transfer steps A_3 and A_5 are somewhat slower than in the wild-type (τ_3 = 70 μs and τ_5 = 20 ms instead of 35 μs and 12 ms, respectively). Furthermore, an additional component is observed at τ_4 ~ 3 ms which does not occur in wild-type. The absolute value of the amplitude A_4 amounts to about half of the sum of the negative amplitudes $A_1 + A_2$.

Since interesting anion effects were observed in the photovoltage kinetics of wild-type bR at low pH (Moltke and Heyn, 1995), we have investigated the influence of Cl^- and SO_4^{2-} on D212N. While the chromophore has a purple color below pH 7 in the presence of Cl^-, it is blue when K_2SO_4 is used instead (data not shown) and the pumping activity at pH 5 is almost completely abolished, as demonstrated by Fig. 5 (*trace 1*). Of the four components fitted to this signal, the two with a positive amplitude have time constants of 2 and 20 ms. Exchanging the electrolyte for KCl restores the pumping signal (Fig. 5, *trace 2*). Most importantly, a new component appears at 70 μs and the 20 ms relaxation grows in amplitude. These effects are more pronounced at higher KCl concentrations (data not shown). The harsh procedure of exchanging the electrolyte solution in the cuvette also has a minor effect on the system discharge time constants, but this does not affect our conclusions.

Fig. 4 *C* shows a possible decomposition of the photovoltage from D212N at pH 5 in KCl into two components. One corresponds to the signal as measured in sulfate and the other to net proton transfer as measured with wild-type. Assuming that the fast negative relaxations and the 3 ms component (equivalent to the 2 ms observed in SO_4^{2-}) of the photovoltage kinetics in KCl are due exclusively to the charge transport of the blue form, we simulated the complete voltage kinetics of the blue component by adding to these three a fourth relaxation at 20 ms, which returns the signal to the baseline (Fig. 4 *C, trace 2*). Subtracting this "blue signal" from the measured photovoltage gives trace 3 of Fig. 4 *C*, which contains all of the 70 μs amplitude and the remainder of the 20 ms amplitude. The ratio of these amplitudes is ~1:2. Trace 3 matches closely the proton transfer signal of the wild-type at pH 5 (Moltke and Heyn, 1995). The wild-type has a small fast negative amplitude, which is neglected in this decomposition. The main wild-type relaxations at 35 μs and 12 ms also have an amplitude ratio of ~1:2. In the wild-type, titration to pH 1 in SO_4^{2-} converts the pumping signal to a photovoltage very similar to trace 2 in Fig. 4 *C* (Moltke and Heyn, 1995). For wild-type the sum of the absolute values of the negative amplitudes of the low pH signal amounts to slightly more than the 35 μs positive amplitude at pH 5, while the corresponding ratio for D212N at pH 5.1 is $(A_1 + A_2):A_3$ = 2:1. Therefore, we may interpret the photovoltage kinetics of D212N at pH 5.1 in chloride as the superposition of signals from an inactive "blue" species and an active proton pumping species with relative contributions of ~2 to 1.

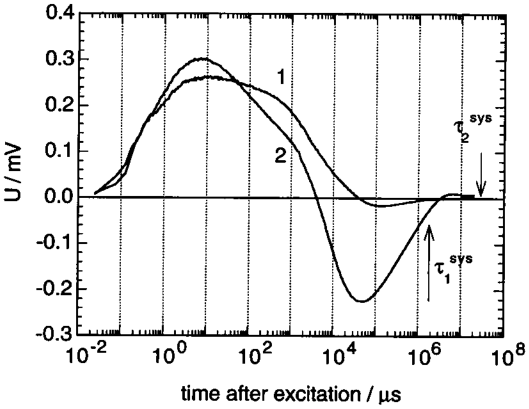

FIGURE 5 Photovoltage kinetics of D212N at pH 5 in standard buffer with 75 mM K_2SO_4 (*curve 1*) and after exchange of the electrolyte with a solution containing 1 M KCl (perfusion of two times the cuvette volume; *curve 2*). The pH was adjusted with H_2SO_4. The signals are not scaled. Almost no pumping, i.e., charging of the membrane before the onset of the system discharge, is observed in K_2SO_4. Addition of KCl restores the signal shown in Fig. 4 *B*. The signal measured in K_2SO_4 consists of four major components with time constants τ_1 = 0.1 μs (bandwidth limited), τ_2 = 1.5 μs, τ_3 = 2.0 ms, and τ_4 = 20 ms. The amplitudes A_3 and A_4 are approximately equal.

The onset of the net charge transport in the presence of KCl can be characterized more completely by looking at the pH dependence of the relative amplitudes. In the signal from the purple state of D212N between pH 4 and pH 7, the relative amplitude of the fast negative processes (A_1, A_2) is diminished compared with the inactive blue forms, while the amplitude of the last active millisecond component (A_5) is increased. This is demonstrated quantitatively in Fig. 6. The pH region of net charge transfer can be clearly distinguished, and the pH dependence of the amplitudes can be fitted with the *Henderson-Hasselbalch* equation for a protonation reaction. Coming from the high pH regime, the active state is produced with an apparent pK_a of 7.2 and vanishes with an apparent pK_a of 3.8.

The effect of chloride outside the pH region from 4 to 7 is not as pronounced but is nevertheless distinctive. Fig. 7 shows that at pH 11 the decay of the photovoltage back to the baseline is faster in KCl (Fig. 7, *curve 2*) than in K_2SO_4 (Fig. 7, *curve 1*). A satisfactory fit of the kinetics in K_2SO_4 requires one positive relaxation with a broad distribution of rates at 480 μs, whereas two distributions with positive amplitudes at 28 and 360 μs are needed in the presence of chloride. Qualitatively the same observation is made below pH 3 (data not shown). Whereas the decay of the signal toward the baseline is described by one distribution of time constants at 8.3 ms in K_2SO_4, two distributions with smaller times of ~100 μs and 2.7 ms are needed in the presence of chloride. This Cl^--induced change of the photovoltage kinetics is quite similar to the difference between the electri-

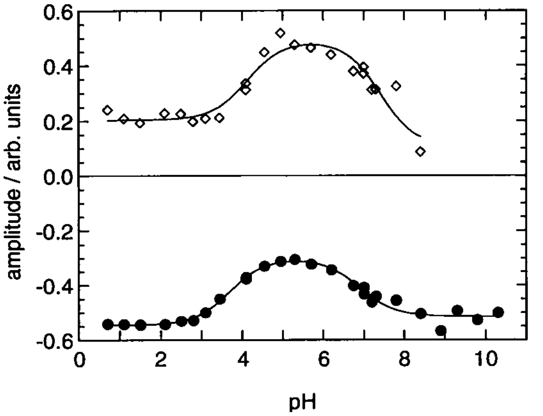

FIGURE 6 Normalized amplitudes $A_1 + A_2$ (●, both of negative sign) and A_5 (◇, the last positive component in the signals, corresponding to an active charge translocation) from D212N in standard buffer with 150 mM KCl at 25°C. The normalization computes the ratio of the respective amplitudes and the sum of the absolute values of all the amplitudes contained in the signal except for the passive system processes. The solid curves represent a fit using the Henderson-Hasselbalch equation for two transitions. The respective pK_a values are: 3.7 ± 0.1 and 6.9 ± 0.2 for $A_1 + A_2$ and 4.1 ± 0.2 and 7.4 ± 0.3 for A_5. Since the absolute signal amplitude decreases with increasing pH, the error for the higher pK_a is largest.

cal signals from the acid-blue and acid-purple states of the wild-type protein (Moltke and Heyn, 1995), although in this pH region the chromophore color is blue for both types of anions.

Between pH 7 and pH 10 (Fig. 4 *A*), neither net transport of charge nor any significant anion effect can be observed. The signal differs from the photovoltage associated with the acid-blue and acid-purple form of wild-type in the larger number of components with positive amplitude and in the fact, that the last charge translocation step occurs at ~100 ms.

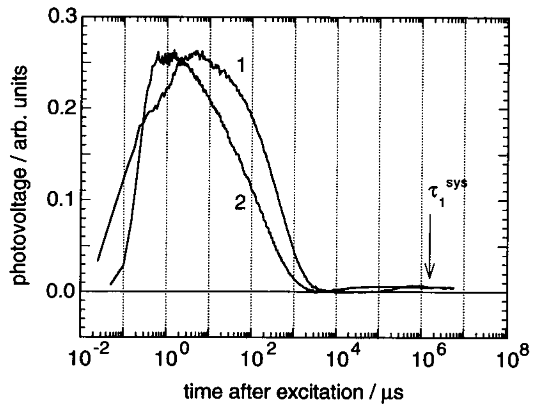

FIGURE 7 Comparison of the scaled photovoltages for D212N at pH 11 (25°C) in standard buffer with 75 mM K_2SO_4 (*1*) and 150 mM KCl (*2*), respectively. The pH was adjusted with KOH. The positive decay of the voltage can be fitted with just one Gaussian distribution of time constants in the case of K_2SO_4 (τ = 480 µs), while two distributions are needed for the chloride-dependent signal (28 and 360 µs).

DISCUSSION

Asp85→Asn

Time-resolved photovoltage measurements for D85N were first reported using *Escherichia coli* expressed and purified mutant proteins reconstituted in vesicles containing *H. salinarium* lipids (Otto et al., 1990). At pH 6 in 150 mM KCl, a signal was observed that was qualitatively similar to that seen in Fig. 2. A large unresolved charge displacement in the direction opposite to that of net transport in the wild-type was followed by a decay toward the baseline in ~10 ms. It was concluded that no net proton transport occurs and that the photovoltage signal is similar to that of the acid blue form of wild-type (Otto et al., 1990). We have focused here on the pH and anion dependence of the photovoltage of the D85N mutant expressed in *H. salinarium* and purified in purple membrane form (Krebs et al., 1993). The isolation of the mutant in large membrane patches greatly improved the adsorption of the membranes to the support. Together with the improved time resolution of the experimental setup, this led to a much higher signal-to-noise ratio.

The data of Fig. 2 show that at pH 7 in 150 mM KCl the photovoltage of D85N differs from that known for the acid-blue form of the wild-type (Moltke and Heyn, 1995) by the additional positive and negative components at 60 µs and 1.3 ms, respectively. However, at pH 6 the observations made earlier (Otto et al., 1990) are confirmed (data not shown). The photovoltage signal at physiological pH seems to be a superposition of the wild-type acid-blue signal and this positive/negative pair of relaxations. This pair is absent at low pH and appears only above pH 6.5. It seems to be associated with the species absorbing at 615 nm. Similar features were observed in the photovoltage kinetics for the mutant D85A (data not shown) and in the integral of the photocurrent kinetics of D85N at pH 7 (Gergely and Váró, 1992), where only the 60 µs component could be discerned. The electrical components at 60 µs and 1.3 ms are nicely correlated in time with the rise (43 µs) and decay (1.3 ms) times (U. Alexiev, M. P. Krebs, R. Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results) of the long-living L intermediate in the photocycle at pH 7, which is typical for the acid-blue form of bR. The 60 µs time is close to the proton release time of 71 ± 4 µs observed with a surface-bound pH indicator dye on the extracellular surface at position G72C (Alexiev et al., 1995) and is comparable to the 85 µs electrical relaxation time from current measurements associated with the rise of M in wild-type (Liu, 1990). As already observed for D85N in DMPC/CHAPS micelles (Otto et al., 1990), proton release and uptake in D85N purple membranes, as measured with pyranine in the bulk phase, occur in the normal sequence (Kataoka et al., 1994). Therefore, we interpret the approximately equal positive and negative electrical amplitudes at 60 µs and 1.3 ms in the following way. A structural change associated with the L intermediate lowers the pK_a of the releasing group XH (Zimányi et al., 1992) such that it transiently deprotonates to the extracellular surface. The proton is then taken up again by this group during the decay of L. Thus, the structural change that induces the pK_a drop of XH to between 6 and 7 occurs in the absence of M. This is analogous to the transient deprotonation of aspartate 96 and proton release to the bulk observed in the blue form of D212N, which also lacks an M intermediate (Cao et al., 1993). In the acid-blue form of bR at very low pH, this transient deprotonation of XH is not observed, because the induced drop in the pK_a is not large enough and XH remains protonated.

While at pH 7 there is no difference between the photovoltage in chloride or sulfate (Fig. 2), we observe the same difference as between the acid-blue and acid-purple form of the wild-type, if we lower the pH to 1 (Fig. 3). The chloride-induced transition between the electrical signals characteristic for the acid-blue and acid-purple states still occurs in the mutant D85N at low pH, but surprisingly is not observed at neutral pH. One possibility is that the chloride binding is much weaker at neutral pH due to the presence of the negatively charged aspartate 212. Evidently, the protonation or neutralization of aspartate 85 is necessary but not sufficient for the formation of an acid-purple-like state of the protein.

Replacement of aspartate 85 by the neutral residue asparagine reduces the pK_a of the SB to ~7 and an M-like state with a deprotonated SB is detected in the dark at very alkaline pH (Otto et al., 1990; Marti et al., 1991, 1992). Further studies have shown that this transition is more complex and that a purple state coexists between the blue form (neutral pH) and the high pH yellow form (Turner et al., 1993; Brown et al., 1993). The pH-dependent equilibria in the dark were interpreted as transitions between an O-like blue form absorbing at 615 nm, an N-like purple form absorbing around 570 nm and the M-like yellow form absorbing at 405 nm (Turner et al., 1993). We find pK_a values of 8.2 and 9.6 for these complex titrations (data not shown). At pH 10.8 (Fig. 1) we estimate from our titration data that our sample was 20% in the N state and 80% in the M state, in good agreement with previous results (Turner et al., 1993). Only the N-like population will be excited with the excitation wavelength of 580 nm used for the photovoltage measurements. Accordingly, the small amplitude of the electrical signal at pH 10.8 is not surprising and due only to a small fraction of the bR molecules. The N-intermediate of wild-type is known to have a photoproduct designated variously as R_{660}, K_N, or Q absorbing around 650 nm (Váró and Lanyi, 1990; Yamamoto et al., 1992; Tokaji and Dancsházy, 1992). The photocycle of D85N at pH 11 is indeed dominated by one major intermediate at about 650 nm rising with 2.7 ms and decaying with 54 ms (U. Alexiev, M. P. Krebs, R. Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results). These photocycle data thus support the idea of an N-like initial state. At 410 nm the absorbance changes were very small, and no evidence for an M intermediate was found, in agreement with the results for the wild-type photoproducts of N (Yamamoto et al., 1992). The electrical data of D85N at pH 10.8 (Fig. 1) were analyzed with three positive amplitudes, the first of which is bandwidth limited. The second and third amplitudes have time constants of 480 μs and 10 ms, respectively. Before the expected system discharge at 1.5 s, there is a further negative component at a few hundred milliseconds, the amplitude of which amounts to only one fourth to one fifth of the positive amplitudes. Aspartate 96 is presumably deprotonated in this N-like conformer. At pH 10.8 one is very close to the pK_a of aspartate 96 in the dark (Száraz et al., 1994). Thus, one explanation for our electrical data may be the transient reprotonation and deprotonation of aspartate 96 from the cytoplasmic surface. However, the positive and negative amplitudes should then be equal unless the photocycle is slower than 1 s (the time constant of the passive system discharge). Photocycle measurements indicate a return to the initial state in 54 ms (U. Alexiev, M. P. Krebs, Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results), i.e., well before the system discharge. The observation of the system discharge in the photovoltage kinetics thus indicates a net charge transfer up to this time. Using the dye alizarin yellow at pH 11 and measuring the absorption changes at 490 nm, proton uptake and release times of 4.4 and 57.5 ms were observed (U. Alexiev, M. P. Krebs, Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results). Therefore, the main charge displacements at 480 μs and 10 ms can be interpreted as net transport in the same direction as in wild-type with proton uptake preceding proton release. The meaning of the negative photovoltage at 100 ms as well as the source of the proton remain unclear, because the SB does not deprotonate as shown by the lack of an M intermediate.

Net charge transport by D85N has been observed under continuous light conditions around pH 7 using steady state current measurements, but only when blue light ($360 < \lambda < 420$ nm) was used and not in the presence of yellow light ($\lambda > 515$ nm) (Tittor et al., 1994). This means that the M-like species, which is already present at pH7, is active, whereas the N-like species only accumulates at higher pH. It is highly improbable that the species having a deprotonated SB and absorbing at 410 nm was excited by the 580 nm laser flash used in our experiments. Therefore, we have to attribute our high pH signal to the N-like conformer and it must be concluded that both this and the M-like species are active ion transporters.

Asp212 → Asn

For the bR mutant D212N our photovoltage measurements with sulfate as an electrolyte show an electrical signal typical for the acid-blue form of bR at every pH (except for the interval between pH7 and 10). More importantly, there is no net proton pumping at any pH. The red-shifted absorption maximum of the chromophore supports this interpretation. Measurements of the proton pumping activity in vesicles at pH 7.2 in 1.5 M SO_4^{2-} also suggest a lack of transport (Cao et al., 1993). In the presence of 150 mM KCl, the proton pumping is partially restored in the pH interval between 3.8 and 7.2 (Figs. 4–6). Outside this range the photovoltage resembles that of the acid-purple form of bR, again except for the interval between pH 7 and 10. Fig. 5 shows this chloride-induced activation from an inactive blue form in sulfate to an active purple form at pH 5.

In the Results section, we proposed a decomposition for the photovoltage of D212N in chloride at pH 5 (Fig. 4 *C*), which leads us to several conclusions. First, the agreement of the kinetics for the active component (Fig. 4 *C*, *trace 3*) with the wild-type time constants and amplitudes at neutral pH suggests that the pumping mechanism is similar in both cases. The charge transfer times of 70 μs and 20 ms are thus probably associated with the rise of M (67 μs) and the rise of O (54 ms), respectively, which were observed for D212N in suspensions at pH 5 in chloride (H. Otto, M. P. Krebs, R. Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results). Second, the decomposition is compatible with previous measurements of the activity and of the photocycle (Needleman et al., 1991; Cao et al., 1993). The transport activity at pH 5 and 160 mM NaCl in this purple form is only about 30% that of the wild-type (Cao et al., 1993), which is consistent with the observation that also a reduced amount of M is formed (Needleman et al., 1991). The photocycle measurements in NaCl at pH 4 were interpreted using a cycle that branches at the L intermediate (Needleman et al., 1991). A fraction of the cycling population continues along a blue photocycle, which lacks an M intermediate and returns to the initial state via an N intermediate, while the rest returns to the initial state along a purple photocycle via M, N, and O intermediates. However, the photocycle data could be interpreted equally well on the basis of parallel blue and purple photocycles (Needleman et al., 1991). Based on the comparison of the amplitudes of the "blue component" and the "active component" of the photovoltage, we estimate that ~30% of the molecules are in the active fraction. The superposition proposed in Fig. 4 *C* based on parallel cycles is the simplest model to explain our data and is in good agreement with the observations of a reduced amount of M and of lower pumping activity. However, a more complex analysis based on a branched photocycle cannot be excluded.

The halide-induced activation of D212N has pK_a values of ~3.8 and 7.2 (Fig. 6). The higher pK_a correlates very well with the change in λ_{max} from purple to blue for which a pK_a of 6.9 was obtained (Needleman et al., 1991). It is unclear, however, which amino acids are responsible for this transition. The lower pK_a is presumably due to the protonation of aspartate 85.

We attributed the net charge movement observed for D212N in the presence of chloride (Fig. 4 *C*, *trace 3*) to proton pumping on the basis of the close similarity of the kinetics to that of wild-type. Our experiments cannot exclude chloride transport in the opposite direction. Previous measurements of light-induced transport activity in envelope vesicles showed, however, that protons are transported (Needleman et al., 1991; Cao et al., 1993).

In the presence of sulfate as the anion, D212N is inactive at any pH, whereas in the presence of chloride net proton pumping is restored. This anion effect may well be due to the difference in size of these anions. While sulfate may be too large, chloride may be small enough to enter bR and to bind close to the SB, effectively replacing the negative charge of Asp 212. Such a role for chloride as a surrogate counterion had been suggested previously on the basis of titration and halide binding experiments with the double and triple mutants D85N/D212N and R82Q/D85N/D212N (Marti et al., 1991, 1992). In those experiments the λ_{max} value of the chromophore increased with the ionic radius of the halide anion in the sequence F^-, Cl^-, Br^-, I^-, strongly suggesting direct binding to the protonated SB (Marti et al., 1992). With D212N in the presence of chloride, the λ_{max} value of the chromophore shifts from 585 to 570 nm when the pH is lowered below the upper pK of 6.9 (Needleman et al., 1991). Our electrical measurements show that in parallel the proton pumping activity is restored. The direction of the color change is again consistent with chloride binding close to the SB. The chloride binding may shift the equilibrium between inactive and active forms of the mutant in favor of the active form. Alternatively, chloride binding may increase the branching ratio between active and inactive branches of the cycle. The mechanism of activation may involve a small chloride-induced decrease in the distance between the SB and the carboxyl of Asp 85 in the L intermediate. Another possibility is that the negative charge of Cl^- or Asp 212 is required to sufficiently reduce the pK difference between the SB and Asp 85 in the L intermediate.

At pH 8.9, the photovoltage signal has some similarity to that of the acid-blue form of wild-type (e.g., no net charge transfer occurs), but its decay is significantly slower (Fig. 4 *A*). Under these conditions, the photocycle has no M intermediate and the depletion signal is at 610 nm. The L/N intermediate at 490 nm decays with 200 ms together with the return to the initial state (H. Otto, M. P. Krebs, R. Mollaaghababa, H. G. Khorana, and M. P. Heyn, unpublished results). This is in excellent agreement with the slowest photovoltage component at 0.2 s. Measurements using the pH-sensitive dye pyranine with micelles (Otto et al., 1990) and membranes (Cao et al., 1993) showed that in the blue form of D212N protons are transiently released and taken up. With purple membranes, proton release and uptake times of 2 and 160 ms were observed at pH 6.9 (Cao et al., 1993). Time-resolved FTIR spectra showed that Asp 96 deprotonates during the L-to-N transition with a time constant of 146 μs and reprotonates with a time constant of 122 ms corresponding to the recovery of bR (Cao et al., 1993). Taken together, these results were interpreted as being due to the transient deprotonation and reprotonation of Asp 96 to and from the cytoplasmic side (Cao et al., 1993). The apparent delay between the deprotonation of Asp 96 and the appearance of the proton in the bulk phase may just be due to trapping at the surface (Alexiev et al., 1995). The reported time constants for proton uptake and reprotonation of Asp 96 are in excellent agreement with the slowest electrical relaxation at 200 ms (Fig. 4 *A*, pH 8.9; at pH 6.9 the shape of the photovoltage trace is similar). The sign of this positive photovoltage amplitude is also consistent with reprotonation of an internal residue from the cytoplasmic surface. For the proton release to the cytoplasmic surface, presumably from Asp 96, we would expect an electrical component of equal amplitude and opposite sign in the time range of the 146 μs FTIR signal. The trace at pH 8.9 in Fig. 4 *A* shows at best a negative component of only small amplitude in this time range (the flattening between 100 and 200 μs). The present electrical data are thus in agreement with the model proposed by Cao et al., if Asp 96 were only partially protonated in the L state.

In previous studies on the kinetics of photoelectrical signals in D212N it was not realized that anion effects exist (Gergely and Váró, 1992; Moltke et al., 1992). The partial activation of D212N at pH 5 in chloride was already observed in previous photovoltage experiments (Moltke et al., 1992). In photocurrent measurements of D212N at pH7, it was not possible to resolve the slower components properly, and no conclusion could be drawn about the important question of net charge transport (Gergely and Váró, 1992). An effect of chloride on the early photocurrent signal of D212N at low pH was recently reported (Hong et al., 1994). Because of the limitations of the method, no conclusions about net transport could be reached. This finding may be related, however, to our observation of a Cl^--dependent transition at low pH similar to the acid-blue to acid-purple transition of wild-type.

CONCLUSIONS

D85N

At pH <6.5 no net charge transport occurs in D85N. The transient charge motion is identical to that of the acid-blue or acid-purple forms of wild-type depending on whether sulfate or chloride is the anion present.

At pH >6.5, the photovoltage kinetics of D85N has superimposed on the low pH signal a pair of charge transfer steps of opposite sign and approximately equal amplitude with times near 60 μs and 1.3 ms (pH 7). These charge motions are attributed to the transient deprotonation and reprotonation of the proton release group XH. At neutral pH, no anion effects occur.

At alkaline pH, above the pK of the SB, net proton transport occurs from the N-like state of D85N in the same direction as in wild-type.

D212N

No net charge transport is observed for the mutant D212N in the presence of sulfate, but a transient charge movement occurs that is similar to that of the acid-blue form of wild-type.

In the presence of chloride, net charge transport is restored in D212N in the pH interval between 3.8 and 7.2. Outside this range, transient charge motion occurs that is similar to that of the acid-purple form of wild-type.

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