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Chloride- and pH-dependent proton transport by BR mutant D85N

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Abstract

Photocurrents from purple membrane suspensions of D85N BR mutant adsorbed to planar lipid membranes (BLM) were recorded under yellow ($\lambda > 515$ nm), blue (360 nm $< \lambda <$ 420 nm) and white ($\lambda > 360$ nm) light. The pH dependence of the transient and stationary currents was studied in the range from 4.5 to 10.5. The outwardly directed stationary currents in yellow and blue light indicate the presence of a proton pumping activity, dependent on the pH of the sample, in the same direction as in the wild-type. The inwardly directed currents in white light, due to an inverse proton translocation, in a two-photon process, show a pH dependence as well. The stationary currents in blue and white light are drastically increased in the presence of azide, but not in yellow light. The concentration dependence of the currents on azide indicates binding of azide to the protein. In the presence of 1 M sodium chloride, the stationary proton currents in yellow light show an increase by a factor of 25 at pH 5.5. On addition of 50 mM azide, the stationary current in yellow light decreases again, possibly by competition between azide and chloride for a common binding site. The observed transport modes are discussed in the framework of the recently published IST model for ion translocation by retinal proteins [U. Haupts et al., Biochemistry 36 (1997) 2-7]. © 1998 Elsevier Science B.V.

Keywords: Bacteriorhodopsin; BLM; Schiff base; D85N; pH; Retinal

1. Introduction

Bacteriorhodopsin (BR), the most abundant retinal protein from the plasma membrane of *Halobacterium salinarum*, belongs to the family of seven-helical transmembrane proteins and acts as a light-driven proton pump [1]. Light absorption stimulates bacteriorhodopsin into an excited state that thermally decays through a sequence of optically distinct intermediates back to the ground state [2]. As the net result of the photocycle, at physiological conditions, a proton is released in the extracellular medium and another one is taken up from the cytoplasm and, therefore, a proton gradient is generated across the cell plasma membrane (for recent reviews see [3–8]). A three-dimensional model of the bacteriorhodopsin structure [9,10] showed the potential ''proton conducting channel'' subdivided by the chromophore

Abbreviations: BR, bacteriorhodopsin; BLM, black lipid membrane; FTIR, Fourier transform infrared; EC, extracellular channel; CP, cytoplasmic channel; TPT, triphenyltin

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retinal into two half channels: the extracellular channel (EC) – connecting the protonated Schiff base with the extracellular medium and the cytoplasmic channel (CP) – connecting the Schiff base with the cytoplasm. In the conducting pathway, two amino acid residues were shown to play a central role in the vectorial transport of protons through the proton channel, namely the aspartic acids 85 and 96 (D85 and D96) [11–13]. The negatively charged D85 in the EC channel acts as the primary acceptor of the Schiff base proton [12,14] and the protonated D96 in the CP channel acts as the proton donor for the Schiff base [15–18]. Replacement of D85 by the non-ionizable asparagine in the mutant D85N results in substantial modifications of the proton pump activity and absorbance properties. At neutral pH, no proton translocation could be detected following photoexcitation of D85N [11,15,19] and the photocycle kinetics was described by a sequence comprising L and N but no M intermediates [20–23]. An abnormal light-dark adaptation was reported for the D85N mutant as well [24]. The replacement of D85 by N lowers the negative charge of the Schiff base counterion, lowering thus the pK_a of the Schiff base to a value around 8 depending on ionic strength. As a consequence, at physiological pH, a mixture of chromophores absorbing at about 410 nm (deprotonated form) and about 610 nm (protonated form) coexist [25–27]. A thorough characterization of the ground state composition of D85N was carried out by Turner and co-workers [28]. They have shown that D85N is composed of three distinct spectroscopic species in equilibrium over the entire pH range. The conformation of a deprotonated species at higher pH was also demonstrated by resonance Raman spectra [29] and by an X-ray diffraction study of unphotolyzed D85N [27]. Most of the measurements of the electrical activity of D85N were carried out at neutral pH, thus reflecting mainly the behaviour of the 610 nm form with a protonated Schiff base. A pH study of the pumping activity of wild-type and of several mutant bacteriorhodopsins reported that D85N was inactive at all pH values [30]. Nevertheless, recent work showed that on illumination with blue light, proton pumping in the same direction as in the wild-type BR is present. At the same time, upon illumination with yellow and blue light, in a two-photon reaction, an inverse proton pumping could be detected [25,31]. In the presence of 50 mM azide, both currents were drastically increased. These results were obtained by means of electrical measurements on membrane sheets containing mutant bacteriorhodopsin adsorbed to planar lipid membranes. More recently, Moltke et al. [32] reported proton pumping activity of D85N BR at pH 10.8, using 580 nm exciting light, in time-resolved photovoltage measurements on membrane fragments adsorbed to a lipid-impregnated polyethylene support. At lower pH values they could not detect any proton transport. Similar measurements, carried out on the deprotonated ''M-like'' form of this mutant, confirmed the proton pumping activity in blue light [33].

We present here a pH study of the transient and stationary photocurrents elicited by yellow, white and blue light in membrane sheets containing D85N BR, adsorbed to planar lipid membranes. The high sensitivity of the method allowed in addition to the already known transport modes of this mutant the detection of the proton pumping in yellow light in the same direction as in the wild-type BR.

2. Materials and methods

The mutant bacteriorhodopsin D85N was isolated as purple (blue) membranes from the halobacterial strain L33 transformed with an expression plasmid containing the bop D85N gene. The photocurrents generated in the protein containing membranes were measured using the BLM technique. The black lipid films, having an area of 10^{-2} cm^2, were formed in a Teflon cell filled with an appropriate electrolyte solution (1.3 ml for each compartment). The film forming solution contained 1.5% (w/v) diphytanoyllecithin (Avanti, Birmingham, AL) and 0.025% (w/v) octadecylamine (Riedel-de-Haen, Hannover, Germany) in *n*-decane to obtain a positively charged membrane surface [34]. BLM formation was checked by eye and the lipid film capacitance and conductance were electrically determined. The membrane sheets were suspended in the appropriate buffer solution (OD = 5) and sonicated for 1 min in a sonication bath. Then, aliquots of 20 μl were added under stirring to the rear compartment of the Teflon cell containing the same

buffer. Photosensitivity of the system developed in about 40 min, reaching a maximal and constant value. The membrane was illuminated with a mercury lamp (100 W) and the actinic light passed through appropriate filters, including a heat protection filter. The intensity of the continuous light source was up to $2\,W/cm^2$ at the membrane surface. For ''yellow'' and ''white'' light, cutoff filters, $\lambda > 515$ nm and > 360 nm, respectively (Schott, Mainz) and for ''blue'' light a K40 broadband or a narrow band (398.7 nm) interference filter (Balzers, Liechtenstein) were used. Light intensity was measured as previously described [35]. The suspensions on both sides of the black membrane were connected to an external measuring circuit via Ag/AgCl electrodes, separated by salt bridges from the Teflon cell. The current was measured with a current amplifier (Stanford Research System – SR570). To obtain the stationary currents we added the blue–UV light insensitive protonophore 1799 (2,6-dihydroxy)-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptane-4-one (Dr. P. Heydtler, DuPont Nemours), which permeabilized the lipid membrane for protons. This protonophore works optimally in the pH range from 5 to 7 and, therefore, the measurements done outside this range can provide only qualitative information about the stationary currents. On the other hand, the use of the ionophores allows to be avoided the potential non-linear effects due to a possible imperfect orientation of the membrane sheets because in the presence of uncouplers the light-induced potential is kept at a value of zero, i.e. the potential $V_m = -V_p$ is abolished by the protonophores. V_m is the potential across the BLM covered with PM and V_p the potential across the purple membrane [36]. However, experiments with cell envelope vesicles (where the orientation is perfect), showed already the same light-dependent behaviour as with membrane sheets [31], meaning that the extracellular side faces the planar lipid membrane, which proves a rather perfect orientation.

The pH of the bathing solution was adjusted in the range 4.5–10.5 by titration in the cuvette with either 1 N HCl or 1 N NaOH. For further details see [36]. For the spectroscopic measurements a double beam spectrophotometer (Hitachi U-3000) was used.

Throughout this paper, the outwardly directed currents, that is in the direction of the normal proton pumping in wild-type BR, were taken as negative.

3. Results

3.1. pH dependence of transient photocurrents

The permeabilization of the lipid film to protons after the addition of the protonophore 1799 allows at the same time the observation of the transient and of the stationary currents. The experiments were done by illuminating purple membranes adsorbed to the BLM with yellow, blue and white light (considered as the simultaneous application of yellow and blue light). The transient current in white light shows an interesting behaviour when the pH of the sample is raised above 7.2 (Fig. 1(c), (d)). The development of the transient currents indicates clearly the superposition of at least two signals. Above pH 7.6, the biphasic shape comprises a positive and a negative part showing that in white light (yellow + blue) the currents generated in the one photon processes by photoexcitation with yellow and blue light, respectively, coexist. The curve representing the pH dependence of the transient current obtained upon illumination with white light (Fig. 2(A)) has a sigmoidal shape, evolving from positive values (indicating the displacement of protons toward the cytoplasmic side), below a pH around 7.6, to negative values above it.

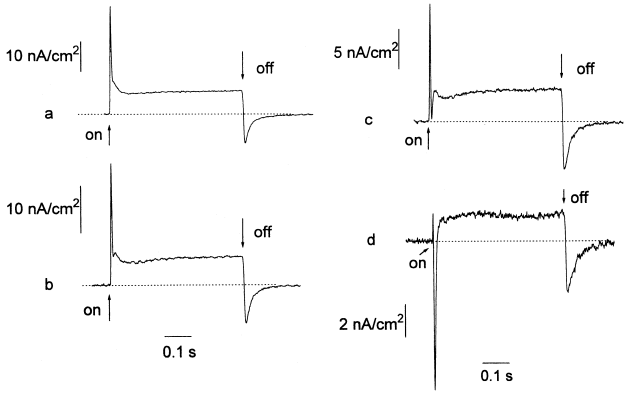

Fig. 1. The currents in white light ($\lambda > 360$ nm, $2.8\,W/cm^2$) at pH 7.0 (a), pH 7.2 (b), pH 7.4 (c) and pH 7.7 (d). The traces indicate two characteristics: the transient and the stationary currents. Altering the pH changes both of them. The conductance of the lipid membrane in the presence of 5 μM 1799 was between 10 and 30 nS. Solution: 100 mM NaCl, 20 mM HEPES. Vertical and horizontal bars indicate the current density and the time scale, respectively.

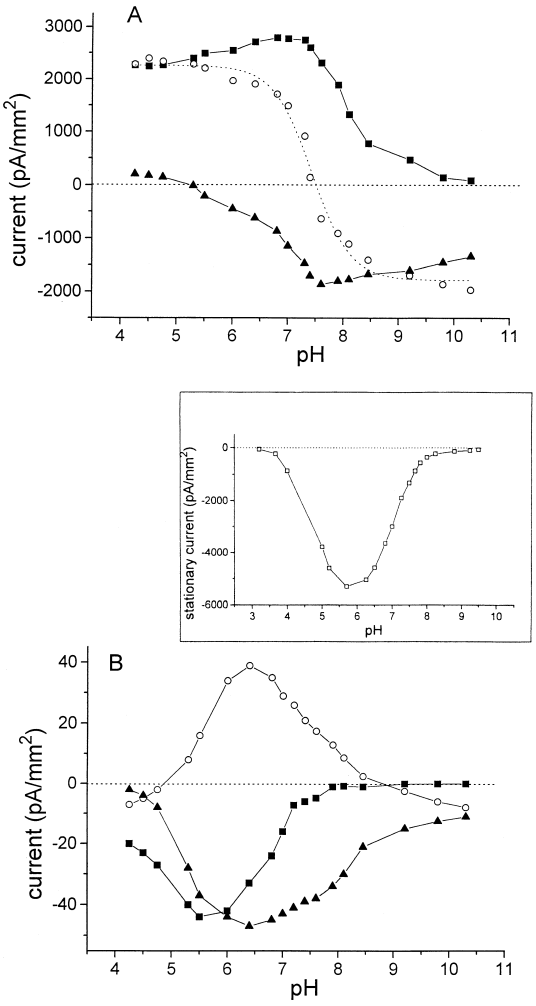

Fig. 2. The pH dependence of the transient (A) and stationary (B) currents. The protein was illuminated by yellow light (solid squares) ($\lambda > 515$ nm, 2 W/cm^2), white light (hollow circles) ($\lambda > 360$ nm, 2.8 W/cm^2) and blue light (solid triangles) ($360 < \lambda < 420$ nm, 0.8 W/cm^2). Inset: pH dependence of the stationary current in wild-type BR, under yellow light illumination. Lines drawn connect the measured values. The dotted line indicates the fit of the curve representing the pH dependence of the transient current upon illumination of the sample with white light to the Henderson–Hasselbalch equation. Experimental conditions as in Fig. 1.

The inwardly directed (that is, toward the cytoplasmic side) transient photocurrent, obtained on illumination with yellow light, has maximal values in the pH range 6.8–7.4. It decreases slightly on decreasing the pH, much more rapidly at pH values above 7 and practically disappears at a pH above 10 (Fig. 2(A)). The size of the transient current can be closely correlated at different pH values with the concentration of the form with a protonated Schiff base (610 nm) [28]. The outward transient current in blue light increases starting from a pH of about 5 to a maximal value at a pH around 8 and then it decreases slightly as the pH becomes higher (Fig. 2(A)). Around pH 5.5 the current becomes biphasic, exhibiting an additional positive component. Below pH 5 the only component remaining is the positive one originating from excitation of the protonated form with blue light.

3.2. pH dependence of stationary photocurrents

The negative stationary currents measured in yellow light (Fig. 2(B), squares) are very sensitive to pH changes and they have significant values only in the range between pH 4.5 and 7. At pH > 8 the stationary currents vanish completely. The stationary currents in blue light (Fig. 2(B), triangles), in the same direction as in the wild-type BR, are present between pH 4.5 and 10 and their values have only slight variations between pH 5.5 and 8. The positive stationary currents in white light (Fig. 2(B), circles), hence in the opposite direction as in the wild-type, have a maximum at a pH around 6.5. The stationary current in white light is the sum of the currents elicited by blue light, yellow light and the one yielded by the two-photon process. The fact that this current becomes negative at higher pH values suggests that the inverse current decreases on increasing the pH of the sample so that, at a pH above 8, the only current observed is due to the one-photon excitation in blue light. The diminution of pumping observed at high and low pH is reversible by titration back to pH 6.8. Similar results were obtained in the presence of 20 mM azide (not shown), except that the stationary currents in white light diminish at a lower pH value (about 7) than without azide. It should be noted that the results concerning the pH dependence of the stationary currents have more a qualitative significance because these currents depend also on the behaviour of the ionophores at low and high pH. Thus, the protonophore 1799 used in our experiments, does not work below pH 3 and it becomes less and less efficient as the pH rises from 7 to higher values. At the extreme pH values this leads to a

limitation of the interpretation of our data. The conductance of the black lipid membrane was checked each time during the whole experiment. Control experiments at 100 mM sodium chloride concentration showed that increasing the conductance by addition of increasing amounts of protonophore leads to larger stationary currents until they reach a saturation value. Our measurements were carried out at a protonophore concentration where the currents are already saturated.

3.3. Titration of the photostationary currents

As already shown [25], the sign and size of the stationary currents obtained with the two photon excitation depend on the light intensity. Here, due to the optimization of the experimental conditions, much larger currents could be recorded without requiring the presence of azide. At pH 6.8, only a small current could be obtained with yellow light, as previously found by Tittor et al. [25], which was considered as negligible in the above cited paper. With additional blue light, an increasing positive current was obtained with increasing blue light intensity (Fig. 3(A)). As the pH was lowered, the negative current in yellow light became larger and when the pH was raised above 7, it disappeared. With blue light alone (Fig. 3(B)), a negative current increased as the pH of the sample increased and this current was diminished with additional yellow light. It reached the zero line as the intensity of the yellow light increased and became positive. At low and high pH, the currents obtained in the two-photon process are smaller, as expected already from the one-photon processes.

3.4. Action spectra

The action spectra for transient and stationary currents were measured with narrow band interference filters, at pH 5.5 (Fig. 4(A),(B)). In order to have comparable conditions of illumination, the light flux was measured in the case of each interference filter, using the same lamp. Then, the signals were normalized so as to have the same photon flux for each wavelength taken into account. The transient currents have negative values in the wavelength range around 400 nm, where the M-like species (having a deprotonated Schiff base) absorbs, and positive ones

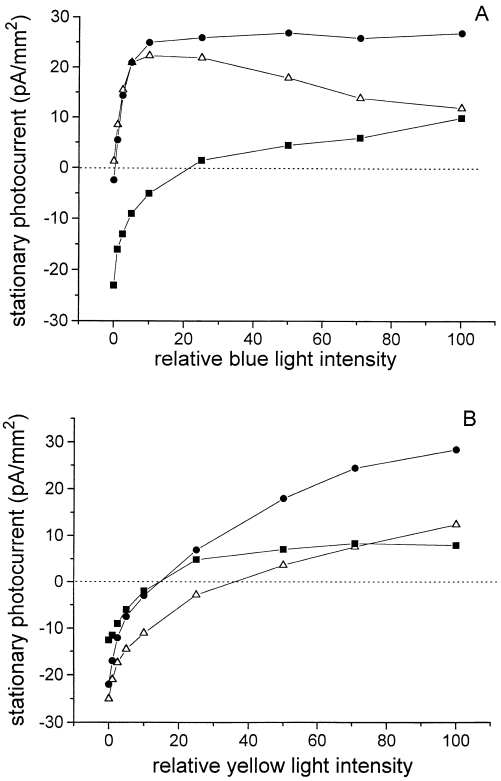

Fig. 3. Titration of the stationary photocurrents with increasing blue (A) or yellow (B) light intensity in the presence of constant yellow (A) or blue (B) light at three pH values: pH 5.5 (solid squares), pH 6.8 (solid circles) and pH 7.6 (hollow triangles). 100% light intensity refers to the intensities given in Fig. 2. The experimental conditions like in Fig. 1.

in the range around 615 nm, where the protonated form is excited. The action spectra for the stationary currents present two maxima, one around 400 nm and the other around 600 nm, indicating that the proton pumping is present also when the exciting light is yellow.

3.5. Effect of azide

A previous paper [25] reported a drastic increase of stationary currents in blue and white light in the presence of 50 mM azide. On the basis of further spectroscopic experiments [26,31], it was demonstrated that, in photostationary conditions, azide accelerates the deprotonation reaction of the Schiff base, as the rate limiting step, in the mutant. In order to determine the half maximal effect, measurements of the stationary currents in yellow, blue and white

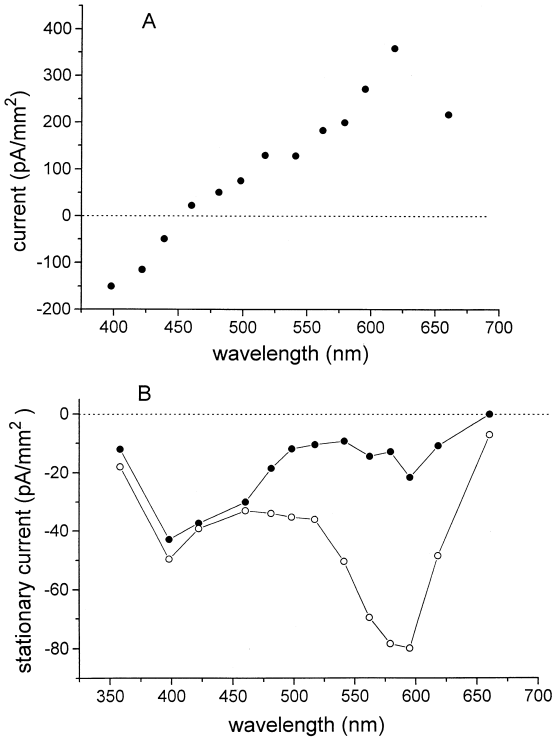

Fig. 4. (A) Action spectrum of the light-induced transient current obtained with a mercury lamp (Osram HBO, 100 W) and different narrow bandwidth interference filters (Balzers, Liechtenstein). (B) Action spectrum of the stationary currents. Conditions: (A) 100 mM NaCl, 20 mM HEPES, pH 5.5; (B) hollow circles: 1 M NaCl, 20 mM HEPES, pH 5.5, solid circles: 100 mM NaCl, 20 mM HEPES, pH 5.5.

light were carried out for increasing concentrations of azide. As already reported [25], the currents in yellow light were hardly affected by addition of azide. The currents in blue light increased as the concentration of azide was raised and this effect was present at several pH values in the range from 6 to 8 (Fig. 5(A)). The increment was larger at a lower pH and decreased at higher pH values. A saturation level was found and the concentration at which it was reached depended on pH, increasing at higher pH values. At pH 6.8, the saturation level was found to be 15 mM, whereas at pH 6 it was 5 mM and at pH 7.6 it was 20 mM. From these data it was found that the binding constant has values between 1 and 2 mM azide, depending on the pH of the sample. The currents in white light showed a different behaviour (Fig. 5(B)). At pH 6.8 the dependence of the current on azide concentration is similar to that found for currents in blue light, except that the increment was much less pronounced (a factor of 3 as compared to a factor of more than 20 in blue light) (Fig. 5(B)). At pH 6, the effect was even smaller and at high pH (over 7.6) the currents became smaller as the concentration increased, reached zero at 20 mM azide at pH 7.6 and became negative at pH 8.5. It should be noted that these data represent the signals measured in white light, not the inverse current, and therefore they show the difference between the inverse current and the current in blue and yellow light. This difference depends on several variables: the pH-dependent relative concentrations of the protonated and deprotonated species, the intensity of light, adsorption of the purple membranes, the conductance of the membrane. Some of them (e.g. the conductance of the membrane, the degree of adsorption of the membrane sheets on BLM) cannot be rigorously controlled from

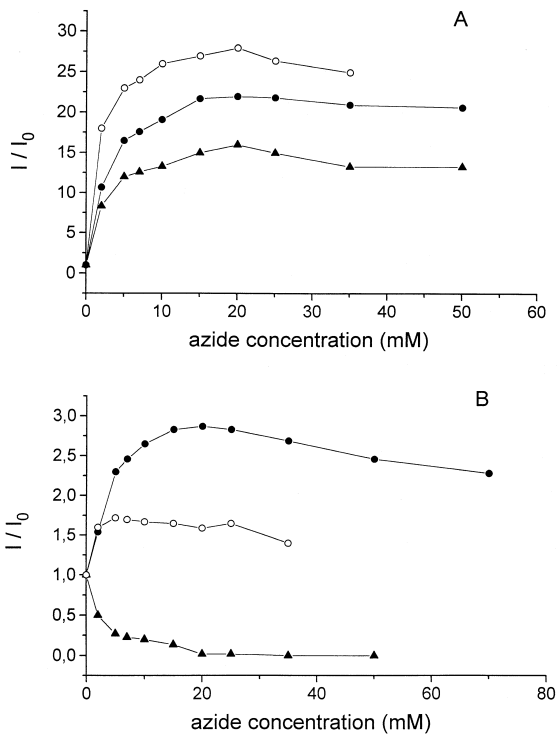

Fig. 5. Effect of azide on stationary photocurrents in blue (A) and white (B) light in the presence of 5 μM 1799 at pH 6.0 (hollow circles), pH 6.8 (solid circles) and pH 7.6 (solid triangles). Lines are drawn only to guide the eye. I is the stationary current at a given concentration of azide. The value I_0 of the stationary current at 0 mM azide is taken as the reference value in all cases. Light conditions are given in Fig. 2. The conditions like in Fig. 1.

one experiment to another. Therefore, it is not possible to have a reproducible quantitative description of the signals with respect to the amplitude, only a qualitative one. In Fig. 5(B), it can be clearly seen how much the effect on this current depends on pH. On the other hand, it seems that the action of azide is stronger for the deprotonated species (as it can be inferred from Fig. 5(B), triangles). Therefore, if there is more of this species in the beginning of the experiment, on adding azide, it will give signals which increase much more rapidly and in this case the difference – that is, the response in white light – will be smaller. Thus, even if the quantitative data are not the same as in a previous report [25] (where a much stronger effect of azide on the currents in white light was reported), due to the different measuring conditions, qualitatively they describe the same behaviour of the system at a similar pH value.

3.6. Chloride stimulated proton currents

The effect of increased amounts of sodium chloride on the transient and stationary currents in yellow, blue and white light was investigated. The experiments were performed at pH 5.5 and the bathing solution contained 100 mM NaCl and 20 mM HEPES. The concentration of NaCl was gradually increased by adding appropriate amounts of concentrated NaCl (4 M). Increasing amounts of sodium chloride led to a substantial increment of the stationary currents obtained with yellow light. The currents were one order of magnitude larger at a concentration of 2 M NaCl. The conductance of the black lipid membrane was checked during the whole experiment and it remained in the range where the currents are at the saturation value. The transient currents recorded upon illumination with yellow light increased slightly as the concentration of NaCl increased, reaching a saturation level around 1 M NaCl (Fig. 6(A)). At pH 5.5 the transient current in blue light has a biphasic shape (not shown) with a positive component (due to the blue light excitation of the protonated form) and a negative one (due to the blue light excitation of the deprotonated form). These two components and the transient current in white light also increase slightly as the salt concentration increases (Fig. 6(A)). The saturation level is again found at around 1 M NaCl. The positive stationary currents in white light decreased as the salt concentration increased, reached the zero line and then acquired increasing negative values (Fig. 6(B)). The small negative stationary currents in blue light increased up to three times as compared to the initial values and seemed to reach a saturation level at about 1.2 M salt concentration. The smaller increase of these currents is probably due to the fact that at this pH there is still some deprotonated form, as can be seen from the biphasic form of the transient current, and its contribution to the signal is not chloride dependent. The large stationary current obtained at pH 5.5 in 1 M sodium chloride, upon illumination of the sample with yellow light, was

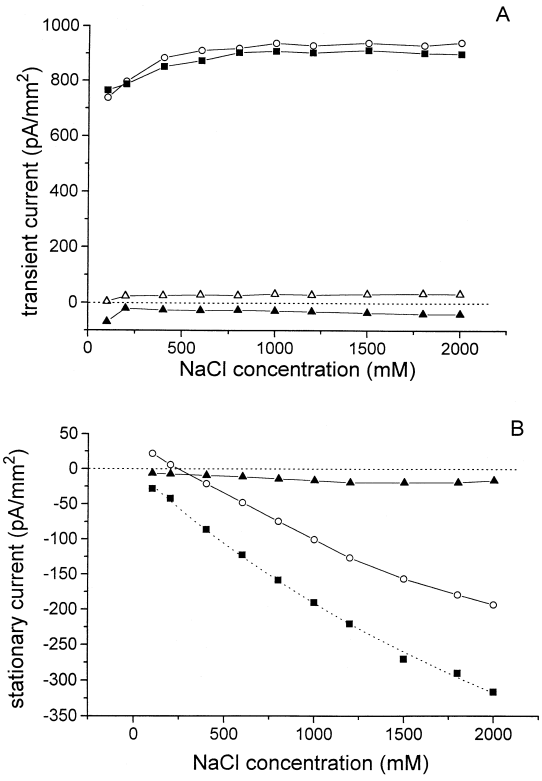

Fig. 6. Effect of sodium chloride on transient (A) and stationary (B) photocurrents obtained with yellow (solid squares), blue (solid triangles) and white (hollow circles) light. The hollow triangles represent the positive values of the biphasic transient currents obtained with blue light. NaCl was added from a stock solution (4 M) to both sides of the membrane to prevent a chloride gradient. The conductance of the lipid membrane in the presence of 5 μM 1799 was between 20 and 90 nS. Conditions: pH 5.5, 20 mM HEPES. Light intensities are given in Fig. 2. The lines drawn connect the measured data points. The dotted line shows the result of a numerical analysis according to the Michaelis-Menten equation and the calculated binding constant is $K_m \approx 3.96$ M.

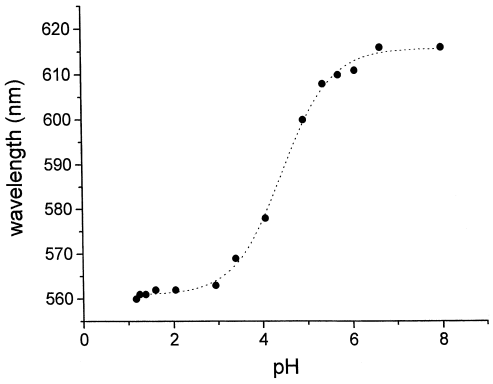

Fig. 7. Titration curve based on the pH dependent blue shift of the absorption maximum of D85N BR in 4 M NaCl. pH was adjusted by adding μM amounts of concentrated HCl. Dotted line: the fit with Henderson–Hasselbalch equation giving an apparent $pK_a \approx 4.5$.

brought back to its value in 100 mM sodium chloride on addition of 50 mM azide. The action spectrum for the stationary currents in the presence of 1 M sodium chloride has a large peak around 600 nm (Fig. 4(B)) where the protonated species absorb. The absorption maximum in 4 M sodium chloride shows a pH dependence and this is shown in Fig. 7. Experiments were also performed using, instead of sodium chloride, increasing amounts of sodium sulfate. No effect similar to that obtained with sodium chloride could be observed in the case of sodium sulfate and the effects were much less pronounced in the case of sodium bromide. Therefore, it seems to be a specific chloride effect and not a simple effect of an increased ionic strength.

4. Discussion

The analysis of the signals measured at different pH values has to take into account the dynamic equilibrium of the species characteristic for D85N BR (O-like, M-like and N-like) and at the same time, the fact that the retinal has different isomeric composition at low and high pH. The all-*trans*/13-*cis* ratio which has a value around 1 at pH 7, in the light adapted form of D85N BR, acquires values > 1 as the pH decreases below 7 and values < 1 as the pH increases above 7 [25,28].

4.1. Transient and stationary photocurrents

At low pH (< 6.5) the protonated BR_{610} form is the only one significantly populated and consequently, the dominant photocycle is the one previously thought to be inactive, which consists of the following steps [21,23]:

$$\overset{h\nu\ (\text{yellow})}{\sim\sim>}\ BR_{610} \rightarrow K \rightarrow L \rightarrow N \rightarrow BR_{610}$$

We found a transient photocurrent in yellow light which is inwardly directed. This shows that a deprotonation of the Schiff base occurs and therefore an M state exists. As the accessibility of the Schiff base is set to the cytoplasmic half of the proton channel [25,27], this transient current corresponds to the movement of protons to the cytoplasm and back. At the same time, a small outwardly directed stationary current (Fig. 8(a)) which is larger at a pH about 5.5 (Fig. 8(b)) and considerably enhanced (by a factor of ten) at high (1 M) chloride concentrations (Fig. 8(c)), indicates the presence of a proton pumping activity, oriented in the same direction as in the wild-type bacteriorhodopsin, for a small fraction of BR_{610}

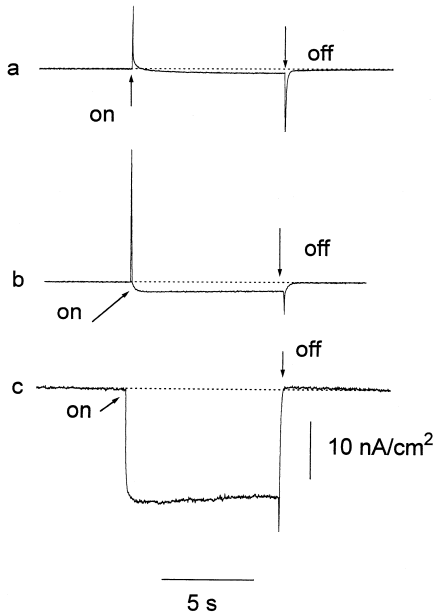

Fig. 8. The stationary currents obtained upon illumination of D85N BR with yellow light alone: (a) 100 mM NaCl, 20 mM HEPES, pH 6.8; (b) 100 mM NaCl, 20 mM HEPES, pH 5.5; (c) 1 M NaCl, 20 mM HEPES, pH 5.5. Light conditions like in Fig. 2. Vertical and horizontal bars indicate the current density and the time scale, respectively.

molecules. This proton pumping activity, though severely reduced in comparison to the wild-type BR, proves the possibility that, in spite of the absence of D85 the wild-type vectoriality of the pump could still be maintained. A molecular dynamic study of D85N BR [37] also suggests the possibility of a proton transfer pathway in this mutant with the same vectoriality as in the wild-type. The direction of the transient current is obviously dictated by the ratio of events leading to proton release at the cytoplasmic side compared to the events leading to proton release at the extracellular side. This will be discussed in more detail below. The behaviour at lower pH is in agreement with the fact that the Schiff base in D85N is largely connected to the extracellular side [27]. The Schiff base proton acceptor in these conditions could be the anionic D212 or some complex involving hydrogen bonding with water molecules [38]. Residue N85 might also participate in such a hydrogen bonding [39]. A control experiment on wild-type BR, illuminated with yellow light, shows a similar pH dependence of the stationary currents, taking also into account the limitations of the efficiency of the protonophore 1799 (Fig. 2(B), inset). The signals in blue light are due to both the deprotonated M-like species, which has an increased concentration as the pH increases, and to the protonated BR_{610} one, which has a correspondingly decreased concentration. Therefore, at all pH values, the transient currents reflect the ratio of the species in equilibrium, representing the sum of the charge motions characteristic for each one. Thus, at a pH around 5, the transient signals become positive, similar to those obtained with yellow light alone, though much smaller. On increasing the pH, the transient currents become outwardly directed, reflecting the increased contribution of the deprotonated species. The stationary currents in blue light, in the direction of the normal proton pumping found in wild-type BR, are at a low pH due mainly to the residual absorption of the protonated form, while at high pH values they are mainly due to the deprotonated form. The differences in the amplitudes, as compared to the same signals in yellow light, can be explained by the difference in the lamp intensities on excitation with blue and yellow light, respectively. In the pH range from 6 to 8, the interpretation of the electric signals is complicated by the fact that they arise as a result of several different photocycles and these photocycles are not independent of each other. In addition to the photocycles of BR_{610}, the photocycle of the M-like form, accompanied by proton pumping, is present as well [25].

Indeed, an FTIR difference spectroscopy study [27] indicates the presence of an M_{cis} intermediate during the photocycle of the mutant, which decays together with the much more abundant N intermediate. At high pH, the equilibrium in the mixture is shifted to the deprotonated species; nevertheless, both the transient and stationary currents in blue light are diminished. This evolution can be correlated with several facts: (a) as the pH increases, the reprotonation becomes rate limiting; (b) at high pH the M-like and the N-like species arise together [27] and the amount of accumulating M depends on the [M]/[N] ratio in the equilibrium mixture; (c) the ratio of the all *trans*/13 *cis* isomers increases at high pH in favour of the latter which is inactive in proton transport [25,28]. These facts, together with a tendency of the protein to denature at high pH values, would explain the diminution of the proton pumping activity. In the case of stationary currents, as already mentioned, the interpretation of the data is limited by the properties of the protonophore 1799 at high pH.

The electric signals, recorded upon illumination with white light are the result of the competition of the one- and two-photon processes. Associated with the two-photon reaction, an inverse proton pumping is present as well [25]. At low pH, where the protonated form is dominant, the transient current has the same direction as the current in yellow light. At high pH its direction is that of the current in blue light. It is interesting to note that this current crosses the zero line at a pH around 7.6 (at this point the two currents, in yellow and blue light, cancel), indicating that the equilibrium between the protonated and deprotonated form is shifted to the deprotonated one. The fit to the Henderson–Hasselbalch equation (Fig. 2(A)) gives a value of 7.6, close to the apparent pK_a of the Schiff base in the mutant D85N BR as it was previously reported [25,28]. The unknown parameters of the photoreactions did not allow the determination of the exact value of the pK_a. The change in the form of the transient signal, as the pH is raised above 7, indicates also a specific behaviour at pH values close to the pK_a of the Schiff base. The biphasic shape with two positive peaks at pH's of about 7.2 and 7.4, turns at

pH 7.6 into a biphasic shape with a positive and a negative peak. The fits with a Henderson–Hasselbalch formula in the case of the signals in yellow and blue light are not so good (not shown) and give pK_a values which differ by approximately 0.5 units from that obtained for the signals in white light. There could be several reasons for these differences: the presence of more than one species in the sample, which have different responses in blue and yellow light, depending on illumination and pH conditions; the intensity of the light was not the same for blue and yellow light and also the parameters of the photoreactions (which are not exactly known) are different in the two cases; the isomeric composition, depending on pH, affects also the electric response; the signal in blue light reflects also a small contribution of the protonated species. In the case of the signal in white light, the conditions are more or less comparable for yellow and blue illumination and are, therefore, more reliable for a quantitative estimation.

4.2. Titration of the photostationary currents

The inwardly directed stationary current in white light reflects even better the competition of the two processes and this can be clearly seen from the curves representing the titration of photostationary currents at increasing light intensities (Fig. 3(A),(B)). In yellow light, a negative current continually decreases with additional blue light of increasing intensity and it becomes positive as the two photon process becomes dominant. Moreover, the pH dependencies of these currents show a close correlation with the pH dependence of the one photon processes. At low intensities of additional blue light, they start from negative values at low pH, in yellow light, whereas at high pH the currents have only positive values. A similar behaviour is found for the currents when, to a continuous constant illumination with blue light, yellow light of an increasing intensity is added. In this case, also in a pH dependent manner, from negative values in blue light alone, the currents achieve positive values as the two-photon process becomes dominant. The pH dependence of the currents in white light indicates a close correlation of the inverse proton pumping with the concentration of the protonated form. This supports the view according to which the inverse current is due to the formation of an intermediate M_{cis} during the photocycle of the all *trans* protonated form. With additional blue light, this M_{cis} will reisomerize to all *trans*, taking a proton from the extracellular half-channel [25]. This will be discussed below in more detail.

4.3. Azide effect

It was shown [26] that in the photostationary state, under continuous blue-light excitation of the deprotonated species of the mutant, a form absorbing maximally at 610 nm is produced which decays in several seconds to the initial state. The decay is accelerated from 4.5 to 1.5 s at pH 9 by addition of azide as the rate limiting step is accelerated and therefore the turnover increases, under light saturation conditions. The existence of a saturation level in the range of several mM azide, which depends on pH, indicates the binding of azide. The fact that azide enhances drastically the stationary photocurrents in blue light suggests that the binding site could be located in the extracellular half-channel, in the vicinity of the Schiff base. In an FTIR study of the photocycle of D96N in the presence of azide, le Coutre et al. [40] have found that during the M to N transition, an azide band at $2040\,cm^{-1}$ disappears and a hydrogen azide band at $2132\,cm^{-1}$ appears. Moreover, the values of the frequencies indicate that the protonation of azide takes place in a hydrophobic region. Only mutation at position 85 can shift the azide band. The authors concluded that the azide might bind closer to D85, in the proton release pathway. Our electrical measurements showed that the action of azide was larger as the pH decreased (Fig. 5(A)), indicating, as already proposed [26], that azide acts in its protonated form.

4.4. Chloride effect

Investigations on wild-type BR at $pH < 3$, where the colour of the membrane turns to blue, revealed that on the addition of chloride anions the membranes almost regained their purple colour [20,41–43]. Resonance Raman experiments on the anion bound form of BR [44] and the alterations found in the photoreactions at acid pH [20], suggested the binding of chloride and other halides in the vicinity of D85. The chloride binding constant increases with decreasing pH [45]. 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 [32]. The increase of the stationary currents in yellow light on increasing the concentration of sodium chloride suggests the possible binding of chloride anions in the vicinity of the Schiff base, although with a very low affinity (the fit with the Michaelis-Menten equation in Fig. 6(B) indicates a $K_m \approx 3.96$ M). We want to emphasize that the stationary current, found at high chloride concentrations, appeared already when the protonophore 1799 alone was added and TPT had no additional effect. Thus, not chloride but protons are the transported ion species. The blue shift of the absorption maximum at high sodium chloride concentration, turning the chromophore to purple ([46], this report), corroborates the results obtained in electrical measurements, indicating again that the chloride anions could bind somewhere in the vicinity of the Schiff base. The bound chloride can function as a counterion to the Schiff base and could, therefore, lower the pK_a of the Schiff base in L ([46], this report). The diminution of the stationary currents in yellow light, suggest a possible competition of the two species for the same binding site, with azide having a higher affinity than chloride.

Our results can be interpreted in the framework of the recently proposed IST model [47]. According to this model, the translocation mechanism by retinal proteins contains three elements: (1) the photoisomerization of retinal from all-*trans* to 13-*cis* (I∗), (2) the conformational change of the protein following the isomerization, the ''switch'' (S), which leads to the modification of the accessibility of the active site of the molecule and (3) the ion transfer reactions within the protein (T) from the active site to the acceptor group and from the donor group to the active site, respectively. The model assumes that the isomerization is followed by a switch and an ion transfer event which kinetically compete. To complete the transport cycle all three steps have to be reversed, leading to minimal six steps to describe a complete transport mode. An individual molecule after excitation (I∗) has the possibility to execute first T or S alternatively, which leads to events of ion transport with opposite vectoriality. Thus, net vectoriality of ion translocation observed in a population is determined by the relative rates of ion transfer reactions and switch. The rates of S and T can be influenced by mutations, light qualities and substrate availability.

In the case of the mutant D85N BR, the lack of D85 as proton acceptor results in several different possible photoelectrical responses. In yellow light, especially at low and neutral pH, where the Schiff base is preferentially protonated, two possible sequences of events, each with a certain probability, can occur after the first isomerization step. Because of the absence of D85, the first T step is slowed down and, therefore, Schiff base deprotonation and switch can compete. Either the Schiff base deprotonation (T) precedes the switch (S) as it is in the case of wild-type – the first case – or the switch (S) can precede transfer (T) – the second case.

The first case describes the *outward proton translocation in yellow light*, with a sequence of events found also in wild-type BR: I∗–T–S–T–I–S. The photoisomerization (I∗) of retinal is followed by the transfer T of the Schiff base proton to the extracellular side before the switch event (S). It remains unclear which group or groups are the acceptor of the Schiff base proton. The *chloride stimulated proton pumping* could be explained by an increased probability of the occurrence of the I∗–T–S sequence, by means of a negative charge located in the proximity of the Schiff base, which may accelerate the deprotonation rate of the Schiff base. In fact, the pK_a decreases with increasing NaCl concentration. The transfer T is followed by the switch S, which sets the accessibility of the Schiff base to the cytoplasmic side and, therefore, the second proton transfer (T) from D96 to the Schiff base can occur. The cycle is completed by the thermal isomerization (I) of retinal followed by the reset of the switch (S).

In the second case, when the switch precedes the Schiff base deprotonation (T), the proton is released to the cytoplasmic side and taken up from the same side after thermal isomerization and the switch step. The sequence of events I∗–S–T–T–I–S describes the *inward transient current* found on illumination with yellow or white light. Note that the two transfer steps are not separated by a switch element. This sequence can, therefore, not lead to a steady-state proton translocation.

If a second blue photon excites the molecule after the I∗–S–T events, a second photoisomerization I∗ leads to the sequence I∗–S–T–I∗–S–T, which de-

scribes the *inverse proton translocation in white light*.

At pH values > 6, where the concentration of the form with a deprotonated Schiff base increases as the pH increases, the outward proton translocation in blue light occurs and it is described by the sequence I∗–S–T–I–S–T. Here the low pK_a of the Schiff base causes the binding site to be unoccupied and, therefore, does not allow a transfer step before the switch. Thus, the switch S precedes the transfer T, which is directed from the cytoplasmic side to the unoccupied active site. After the thermal isomerization I and the reset of the switch S to the extracellular part, the cycle is completed by the last slow transfer T.

It should be noted that most of the present data could be also explained by the conformational equilibrium model [27], according to which there is an equilibrium between two conformations with different accessibilities. In this model the conformational change occurs after the ion translocation, which creates the conditions for the switch. In the case of unilluminated D85N BR, the electrostatic configuration around the Schiff base is to some extent similar to that present in wild-type BR after the proton transfer from the Schiff base to D85. Therefore, the ion translocation is not any more required for the conformational change to occur, because if the equilibrium between the two conformations is not far from the middle from the beginning, the conditions for the switch are already fulfilled, at least for a certain number of molecules. This equilibrium can be shifted by small variations in the local conditions, influenced by pH changes, salt concentration, light intensity etc. The *inward transient current* in yellow light can occur as the result of a conformational change without requiring an ion translocation step before, as the appropriate conditions are already present. The presence of chloride in high concentration sets the electrostatic configuration closer to that found in wild-type BR and allows thus the occurrence of an outward proton translocation on yellow illumination, again with the ion translocation – conformational change sequence. The proton pumping in blue light by the deprotonated form, could be also explained with the ion translocation – conformational change sequence (like in wild-type) if the access for this species would be to the cytoplasmic side.

In order to decide between the two models, the molecular requirements for the transfer and switch processes should be known in more detail.

In conclusion, several proton translocation modes are present in the mutant D85N BR, similarly to mutant D85T BR [48], each with a given probability and the particular way in which the excited molecules behave is determined by the ratio of the rate constants for the ion transfer and switch processes. Our results show that even if D85 is missing, the transport of protons is possible. D85 highly optimizes the vectorial proton translocation. The enhancement of the proton pumping activity in yellow light on the addition of increasing amounts of chloride, suggests that chloride could replace to some extent the role of D85 in the deprotonation reaction of the Schiff base.

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